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Lahoda, Lars-Uwe

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THE ROLE OF LIPOPOLYSACCHARIDE BINDING PROTEIN IN INNATE IMMUNITY IN INFECTED PARTIAL THICKNESS BURNS

LARS-UWE LAHODA

THE ROLE OF LIPOPOLYSACCHARIDE BINDING PROTEIN IN INNATE IMMUNITY IN INFECTED PARTIAL THICKNESS BURNS

Lars-Uwe Lahoda

Stellingen behorende bij het proefschrift

THE ROLE OF LIPOPOLYSACCHARIDE BINDING PROTEIN IN INNATE IMMUNITY IN INFECTED PARTIAL THICKNESS BURNS

van Lars-Uwe Lahoda

1. Lipopolysaccharide (LBP) is an integral part of the innate immune system which defends against Gram-negative infections even in partial thickness burns. (dit proefschrift)
2. With regard to innate immunity, LBP-knockout mice profoundly increase intradermal neutrophil chemoattraction in partial thickness burns when challenged by Gram-negative bacteria. (dit proefschrift)
3. Adenoviral gene therapy applied in partial thickness burns of small rodents is feasible. (dit proefschrift)
4. PG-1, an antimicrobial peptide which is part of the innate immune system, is biologically not deactivated when mixed with a fibrin glue and applied onto an infected partial thickness burn, and can therefore be considered for burn wound treatment. (dit proefschrift)
5. Notwithstanding all progress in the treatment of infections, in 2012 the classical statement "Ibi pus, ubi evacua" still holds firmly.
6. Shoot for the moon. Even if you miss, you'll land among the stars. ~Les Brown
7. Nothing is more treacherous then the obvious.
8. What the superior man seeks is in himself, what the small man seeks is in others. ~Confucius
9. Extreme remedies are very appropriate for extreme diseases. ~Hippocrates
10. Live in New York City once, but leave before it makes you hard; live in Northern California once, but leave before it makes you soft. ~Mary Schmich, Baz Luhrmann - *Everybody's Free (To Wear Sunscreen)*
11. Most researchers use statistics the way a drunkard uses a lamp-post – more for support than illumination. ~Winifred Castle

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Chapter 1

General Introduction

1.1. General Introduction

1.1.1. Fire and the human body

Fire is one of the oldest achievements of mankind. It has brought warmth, light and enabled to process materials. It has also made cooking possible and therefore aided in the preparation as well as preservation of food. It can be stated that fire has significantly contributed to major evolutionary steps. Along with lots of benefits, there was always the danger of an accident; the destructive nature of fire and the danger of being burnt are imminent since ancient times. Burns historically originate from social, environmental and industrial causes. Today, at least in the western world, they are largely outnumbered by accidents originating from means of transportation as well as warfare or catastrophes.

Our skin has many different functions: it protects us and warns us if we are getting too close to a heat source. Intact skin represents a barrier. It protects mechanically, thermally and chemically, it shields (to some extent) against radiation and prevents bacterial invasion. Skin regenerates by itself throughout life, it even lubricates itself and contains several different types of glands and appendices such as hair and nails. Next to these functions this organ plays an important immunologic role by balancing fluids, proteins and electrolytes, it supports renal function (*'perspiratio insensibilis'*) and is crucial for thermoregulation. Endocrine capacity leads to Vitamin D processing and last but not least, skin represents somatovisceral sensibility, a fact that not only the vision-impaired acknowledge and know too well.

Heat is capable of harming the human body in several ways, locally, and systemically. High temperature triggers pain and alerts the organism, denaturizes proteins and as a consequence destroys the intact layers of the protective skin in varying depth, depending on temperature and time of exposure. Burns are graded in I, IIA and IIB, III and IV degrees of burns, with I being defined as erythema and IIA a superficial 2nd degree of burn with blister formation due to a destruction of the epidermis, damage which will restore within 2 weeks' time. IIB stands for a 'deep' intradermal burn and is characterized by a viable wound bottom, from which the wound may heal, and a III degree of burn represents a full thickness, dermal burn, where all dermal structures have been destroyed and healing can only occur form the wound edges and deeper tissues if left untreated. In addition to this classic system, a 4th degree was added over time and meaning an even deeper destruction and exposure of functional tissues such as muscle, tendons and bone.
Superficial burns (I and IIA) heal without scars if uninfected, deeper burns bear an 'eschar' (a layer of dead dermal tissue) of varying thickness. As such, the deeper the burn wound reaches, the longer and more challenging it gets for the body to deal with and

ultimately heal in a scar. Untreated deeper burn wounds are to be avoided due to unfavorable outcomes, such as poor aesthetics as well as poor function. This may lead to unstable coverage of the underlying tissue resulting in impairing contractures.

1.1.2. Burn wound infections

Immediately after the injury, burn wounds are initially sterile. Sterile wound coverage and dressing changes and immediate treatment help prevent further infection. Unfortunately, over time the transient Gram-positive bacteria naturally colonizing the surrounding undamaged skin, as well as Gram-negative bacteria (normally not found on healthy and intact skin) invade the burn wound if untreated. The longer the skin is not capable of completely healing and restoring its protective layers, the higher the bacterial and sometimes fungal numbers found. *Vice versa*: the more bacteria and / or fungi are present in burns, the more complicated, less successful and more time-consuming the healing process becomes, and the weaker the host gets.

Infected burn wounds are a source of high morbidity and mortality (1). In the US the lethality arising from burns in males is roughly 4%, whereas in females it is 5.5% (2). Deaths related to burns increase with advancing age, burn size and the presence of an inhalation injury (3). In 2010, 450,000 medical treatments resulting from burns led to 45,000 hospitalizations and on average 5,000 extensive surgical reconstructions and interventions (3). The top complications following burns recorded in 126,000 complications according to the report of the American Burn Association are in descending order: pneumonia, urinary tract infection, wound infection, respiratory failure, and septicemia. Currently, sepsis mortality originating from either burn, trauma or other conditions leading to an intensive care treatment, reaches numbers between 37% on general intensive care units and 65% on burn units (4).

Over time, bacteria developing multi-resistance have become an increasing problem in such surroundings. Burn wound infections are possible sources of sepsis, which pose serious threats to patients, and represent major challenges to the treating physician. Gram-negative bacteria are challenging to treat and eradicate (5) due to a weakened host that lacks competing, opportunistic Gram-positive bacteria. They favor nutritious wound fluids and moist dressings needed in burn care. Due to their nature, Gram-negative bacteria form 'biofilms', meaning they stick to surfaces and surround themselves with a mechanically protecting protein barrier, making antibiotics hard to penetrate. Routinely, antibiograms are taken repetitively and frequently show rapid changes in antibiotic sensitivity and –resistance, leading to challenging and difficult therapies (6).

1.1.3. Lipopolysaccharide binding protein (LBP) as part of the innate immune system

Next to the mechanical barrier of the skin acting as a shield, the host has limited options to address the problem of invading bacteria in case this shield is breached. It is most important for the immune system to generate a quick response to the threat. A key component of the innate immune system dealing with Gram-negative bacteria is lipopolysaccharide binding protein (LBP). LBP is a glycoprotein, synthesized as a 50 kD polypeptide at numerous sites of the organism such as the lung, the liver, the gastrointestinal tract, and the skin. It is present in serum at a concentration of 2-4 μg/ml and rises as an acute phase protein in case of infection (7). LBP potentiates bacterial killing mediated by bacterial permeability increasing protein (BPI) (8) by 10,000-fold, it disaggregates lipopolysaccharide (LPS), enhances the binding of LPS to the cellular membrane via the (soluble and membrane-bound) CD-14 complex (9), and binds to and therefore activates Toll-like receptor 4 (TLR-4) (10). This protein also potentiates the production of tumor-necrosis-factor α (TNF-α) by macrophages, and according to several investigators, it protects mice from lethal bacterial peritonitis in a dose-dependent manner (11-13). Furthermore, its messenger RNA-levels rise in abdominal sepsis, indicating a key role of LBP in Gram-negative sepsis (13). Lipopolysaccharide (LPS) of Gram-negative bacteria acts as an inducer of the innate immune system via TLR4 and myeloid differentiation factor 2 (MD-2) (14, 15). LBP and CD-14 seem to play a key role in initiating the pro-inflammatory cytokine production following LPS- TLR-4 and MD-2 activation (16). LBP plays therefore a key role in physiologic and pathophysiologic Gram-negative infection (17).

1.1.4. Antimicrobial Peptides

In recent years, no new classes of antibiotics have been reported while, as stated before, multi-resistant bacteria more and more pose a serious problem in patient care. As a consequence, researchers turned to evolutionarily ancient weapons, such as 'antimicrobial peptides' (AMPs). Their widespread distribution throughout the animal and plant kingdoms suggests, that antimicrobial peptides have served a fundamental role in the successful evolution of complex multicellular organisms (18). Animals and plants possess these potent, broad-spectrum antimicrobial peptides which are used to fend off invading microbes, including bacteria, viruses, fungi and protozoa (18). AMPs are naturally occurring cationic peptide molecules with broad antimicrobial activity. They consist of a hydrophobic and hydrophilic 3-dimensional structure enabling binding and integrating into cellular membranes (19). By binding, subsequently 'pores' are formed, resulting in cellular death (20). There are two major families known to be important in the skin of mammals, namely cathelicidins and defensins (21). The group most widely studied are the cathelicidins, Pro-

tegrin-1 (PGI, PG-1) being one of the major representatives (22, 23).In mammals however, these classes of peptide molecules are encoded by several varying genes (24). Interestingly, they are expressed on epithelial surfaces, produced by immune-competent cells, and are therefore suggested to be part of the innate immune system (25). It is believed that these naturally occurring peptide molecules are active against bacteria, fungi and enveloped viruses, and therefore play a crucial role in mounting an innate immune response to injury and microbial insult (26, 27). Furthermore, they are not only profoundly responsible for antimicrobial activity but also act as signaling molecules activating host cell processes involved in immune defense and tissue repair (21). Recent articles more specifically report that the function of these peptides is not only in direct defense of invading bacteria, but rather do they play a complex role in the immune response not only restricted to the innate immune system (28). Further evidence suggests that they are acting in a concentration-dependent manner (29), and they are described to play a role in infectious skin diseases, dermatitis conditions and several other diseases such as Crohn´s (30-32). AMPs are thought to play a role as mediators of inflammation, and to influence cell proliferation and wound healing as well as cytokine / chemokine production and chemo attraction (33, 34).
Given the potent and broad-spectrum activity of these naturally occurring antimicrobial peptides, which can also be called host defense peptides (HDPs), the idea of using them as novel therapeutics supporting and maybe enhancing the traditional antibiotic spectrums, is not far-fetched (26, 35, 36). There is a definitive need for the development of new classes of antimicrobial agents, especially with regards to problematic wounds as represented by burns.

Summarizing, the clinical dilemma of infected burn injuries presents itself with a wide variety of problems. It is beyond any doubt that burns *per se* represent a problematic injury to the body. Burn wound infection is a source of high morbidity and mortality. More specifically, within infections as such, Gram-negative ones pose a more serious problem than Gram-positives. In recent years it was noted that multiresistant bacteria are on the rise and taken responsible for prolonged hospital stays, higher health care cost, and increased mortality, particularly, when initial antibiotic therapy does not provide coverage of the causative pathogen (37).
Current knowledge arising from *in vivo* experiments with regards to LBP is drawn from abdominal sepsis models, whereas basic science and research models mostly investigate LBP-LPS interaction *in vitro*. To date, little is known about the effect of living Gram-negative bacteria on the immune system in partial thickness burns *in vivo*.
Still, questions like: "…do increased LBP levels in burn wounds decrease numbers of Gram-negative bacteria locally?", "…is there possibly a systemic consequence and which?" and

ultimately: "...does LBP really play an essential role in the innate immune system fighting against invading Gram-negative bacteria in burns?" need to be answered.

1.2. Aim of the thesis

Based upon the knowledge summarized above, the objective of this research was to gain more insight into the role of the innate immune system in infected burn wounds with special regards to LBP-LPS interaction. To date, the interaction of living Gram-negative bacteria in a *per se* problematic deep partial thickness burn wound with the innate immune system is rather unclear. Therefore following aims are defined:

1) *To develop an in vivo, deep partial thickness (IIB) burn wound model in mice and apply this to both a new LBP-knockout mouse model and its corresponding wild type control;*

2) *To gain more insight into the ability of LBP to reduce the number of living, multiresistant, Gram-negative bacteria in deep partial thickness (IIB) burn wounds with special regards to innate immunity;*

3) *To apply an adenoviral construct encoding LBP as a tool to study whether this intradermal adenoviral gene delivery system represents a feasible therapeutic approach to bacterial infected burns;*

4) *To test the ability of a new class of antimicrobial agents (AMPs) capable of reducing the number of bacteria in our deep partial thickness burn wound model.*

1.3. Outline of this thesis

The first set of experiments in **Chapter 2** introduces a deep partial thickness mouse burn model in a strain of C57BL-6 wild types compared to their LBP knockout, weight- and age-matched siblings. In this model, an adenoviral construct containing the genetic information for rat-LBP was used as a vector to deliver LBP intradermally at the site of burn injury. To be able to compare its effect, and following a series of *in vitro* tests, the viral construct was injected in LBP-deficient mice as well as wild-types, the virus was applied following a series of *in vitro* tests. The injection site was the living bottom of the partial thickness burn wound allowing insight into innate immune mechanisms by enhancing LBP expression locally and potentially confirming its importance in innate immunity. In accordance with the clinical scenario, both animal groups were subsequently infected by

1

a defined, multiresistant, living number of *Pseudomonas aeruginosa* bacteria, to mimic Gram-negative infection of a serious kind, feared in patient burn care. This set up proved the feasibility of gene therapy in our model.

Chapter 3 makes use of the developed model described in chapter 2. Aiming to identify the molecular changes induced by burn and infection in either mouse strain, commercially available gene-chip arrays were used to analyze intradermally extracted, pooled RNA/cDNA. Since greatly different bacterial numbers were found on the skin of our two mouse strains, we sought to determine possible altered gene expression patterns explaining such findings. Striking differences over a variety of genes were found and considered significant, when up- or down regulated at least 2-times compared to the standard in either mouse population. The gene which was found to be the most up-regulated, is known to be a chemo attractant of neutrophils, called growth-related oncogene-1 (GRO-1) and represents a link to the innate immune system as such. GRO-1, a CXC-chemokine, can be regarded as the murine analogue to human interleukin-8 (IL-8). This introduces the neutrophil as a possible key component in the findings of different immunity between the 2 strains.

In order to decipher more clearly the role of the neutrophil and other leukocyte subsets in our LBP-knockout animal model over a longer period of time, systemic and topic tests were conducted in either colony. **Chapter 4** describes the results of peripheral blood analyses of the knockout compared to C57BL-6 wild type mice at several different time points following burn challenge and infection. These experiments were designed to find out, whether an altered local or systemic concentration of neutrophils was the case at several time points. Possibly, the animals had a different white blood pool composition of leukocyte subsets to start off with, explaining profound changes in immune response when challenged. Potentially this would also involve pro- and anti-inflammatory cytokine expression which was also focused on in this chapter. In all of our immunologic and molecular biologic tests conducted in this chapter, LBP-knockout and wild-type animals proved to be different.

Finally, a new class of naturally occurring, antimicrobial peptide molecules playing a role in innate immunity was introduced into our burn wound infection model (**chapter 5**). These peptides have drawn attention due to the fact of their vast variety of antimicrobial activity and their ancient origin (paragraph 1.1.4). In an attempt to use a designer peptide (Protegrin-1, PG-1) in our burn model we conducted a series of *in vitro* and *in vivo* tests to proof its efficacy. Following, this peptides was introduced into our model aiming at answering the question whether it would be de-activated by mixing it with fibrin glue. A mixture of PG-1 and fibrin glue would consequently represent a new topical, antibacterial agent supporting burn wound treatment in case of infection, a great clinical challenge.

The findings of the preceding chapters and the future perspectives related to these findings are discussed in **chapter 6.**

References:

1. Bloemsma GC, Dokter J, Boxma H, et al. Mortality and causes of death in a burn centre. *Burns* 2008;34:1103-1107.
2. American Burn Association Resources Factsheet. Available at: http://www.ameriburn.org/resources_factsheet.php.
3. National Burn Registry Annual Report 2010. Available at: http://www.ameriburn.org/2010NBRAnnualReport.pdf.
4. Mann EA, Baun MM, Meininger JC, et al. Comparison of Mortality Associated With Sepsis in the Burn, Trauma, and General Intensive Care Unit Patient: A Systematic Review of the Literature. *Shock* 2011;[Epub ahead of print].
5. McManus AT, Mason AD, Jr., McManus WF, et al. A decade of reduced gram-negative infections and mortality associated with improved isolation of burned patients. *Arch Surg* 1994;129:1306-1309.
6. Sen S, Greenhalgh D, Palmieri T. Review of burn injury research for the year 2009. *J Burn Care Res* 2010;31:836-848.
7. Schumann RR, Kirschning CJ, Unbehaun A, et al. The lipopolysaccharide-binding protein is a secretory class 1 acute- phase protein whose gene is transcriptionally activated by APRF/STAT/3 and other cytokine-inducible nuclear proteins. *Mol Cell Biol* 1996;16:3490-3503.
8. Elsbach P. The bactericidal/permeability-increasing protein (BPI) in antibacterial host defense. *J Leukoc Biol* 1998;64:14-18.
9. Ulevitch RJ, Tobias PS. Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. *Annu Rev Immunol* 1995;13:437-457.
10. Raetz CR, Whitfield C. Lipopolysaccharide endotoxins. *Annu Rev Biochem* 2002;71:635-700.
11. Zweigner J, Gramm HJ, Singer OC, et al. High concentrations of lipopolysaccharide-binding protein in serum of patients with severe sepsis or septic shock inhibit the lipopolysaccharide response in human monocytes. *Blood* 2001;98:3800-3808.
12. Lamping N, Dettmer R, Schroder NW, et al. LPS-binding protein protects mice from septic shock caused by LPS or gram-negative bacteria. *J Clin Invest* 1998;101:2065-2071.
13. Wang SC, Klein RD, Wahl WL, et al. Tissue coexpression of LBP and CD14 mRNA in a mouse model of sepsis. *J Surg Res* 1998;76:67-73.
14. Park BS, Song DH, Kim HM, et al. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature* 2009;458:1191-1195.
15. Lizundia R, Sauter KS, Taylor G, et al. Host species-specific usage of the TLR4-LPS receptor complex. *Innate Immun* 2008;14:223-231.
16. Tsukamoto H, Fukudome K, Takao S, et al. Lipopolysaccharide-binding protein-mediated Toll-like receptor 4 dimerization enables rapid signal transduction against lipopolysaccharide stimulation on membrane-associated CD14-expressing cells. *Int Immunol* 2010;22:271-280.
17. Schutt C. Fighting infection: the role of lipopolysaccharide binding proteins CD14 and LBP. *Pathobiology* 1999;67:227-229.
18. Zasloff M. Antimicrobial peptides of multicellular organisms. *Nature* 2002;415:389-395.

19. Mihajlovic M, Lazaridis T. Antimicrobial peptides bind more strongly to membrane pores. *Biochim Biophys Acta* 2010;1798:1494-1502.
20. Tachi T, Epand RF, Epand RM, et al. Position-dependent hydrophobicity of the antimicrobial magainin peptide affects the mode of peptide-lipid interactions and selective toxicity. *Biochemistry* 2002;41:10723-10731.
21. Bardan A, Nizet V, Gallo RL. Antimicrobial peptides and the skin. *Expert Opin Biol Ther* 2004;4:543-549.
22. Albrecht MT, Wang W, Shamova O, et al. Binding of protegrin-1 to Pseudomonas aeruginosa and Burkholderia cepacia. *Respir Res* 2002;3:18.
23. Steinstraesser L, Klein RD, Aminlari A, et al. Protegrin-1 enhances bacterial killing in thermally injured skin. *Crit Care Med* 2001;29:1431-1437.
24. Nizet V, Ohtake T, Lauth X, et al. Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature* 2001;414:454-457.
25. Gallo RL, Murakami M, Ohtake T, et al. Biology and clinical relevance of naturally occurring antimicrobial peptides. *J Allergy Clin Immunol* 2002;110:823-831.
26. Braff MH, Gallo RL. Antimicrobial peptides: an essential component of the skin defensive barrier. *Curr Top Microbiol Immunol* 2006;306:91-110.
27. Metz-Boutigue MH, Shooshtarizadeh P, Prevost G, et al. Antimicrobial peptides present in mammalian skin and gut are multifunctional defence molecules. *Curr Pharm Des* 2010;16:1024-1039.
28. Bowdish DM, Davidson DJ, Hancock RE. A re-evaluation of the role of host defence peptides in mammalian immunity. *Curr Protein Pept Sci* 2005;6:35-51.
29. Huang HW. Molecular mechanism of antimicrobial peptides: the origin of cooperativity. *Biochim Biophys Acta* 2006;1758:1292-1302.
30. Schittek B, Paulmann M, Senyurek I, et al. The role of antimicrobial peptides in human skin and in skin infectious diseases. *Infect Disord Drug Targets* 2008;8:135-143.
31. Fellermann K, Wehkamp J, Herrlinger KR, et al. Crohn's disease: a defensin deficiency syndrome? *Eur J Gastroenterol Hepatol* 2003;15:627-634.
32. Wehkamp J, Fellermann K, Stange EF. Human defensins in Crohn's disease. *Chem Immunol Allergy* 2005;86:42-54.
33. Niyonsaba F, Ogawa H. Protective roles of the skin against infection: implication of naturally occurring human antimicrobial agents beta-defensins, cathelicidin LL-37 and lysozyme. *J Dermatol Sci* 2005;40:157-168.
34. Niyonsaba F, Nagaoka I, Ogawa H, et al. Multifunctional antimicrobial proteins and peptides: natural activators of immune systems. *Curr Pharm Des* 2009;15:2393-2413.
35. Zhang L, Falla TJ. Potential therapeutic application of host defense peptides. *Methods Mol Biol* 2010;618:303-327.
36. Ong PY, Boguniewicz M. Investigational and unproven therapies in atopic dermatitis. *Immunol Allergy Clin North Am* 2010;30:425-439.
37. Slama TG. Gram-negative antibiotic resistance: there is a price to pay. *Crit Care* 2008;12 Suppl 4:S4.

Chapter 2

2

Local lipopolysaccharide-binding protein (LBP) adenoviral gene transfer in a rodent partial thickness burn model increases host defense against multiresistant *Pseudomonas aeruginosa*

2

Abstract

Background:
Infections occurring in burns are responsible for high mortality and morbidity. Gram-negative infections are of great concern, but little is known about the complex mechanisms of the innate immune system responsible for eliminating bacteria in partial thickness burn wounds. We aimed at shedding more light onto the role of lipopolysaccharide binding protein (LBP) in clearing bacteria and the resulting innate immune responses in a rodent partial thickness burn model. For this purpose, we used LBP-knockout mice and LBP-rescue experiments initiated by living, multidrugresistant *Pseudomonas aeruginosa* as the source of lipopolysaccharide (LPS).

Methods:
A partial thickness (type IIB) burn wound model was designed in LBP-knockout mice and their wild type control strain (C57BL/6). We developed a rat-LBP encoding adenoviral construct and used adenoviral β-galactosidase (β-gal) as control. *In vivo*, female C57BL/6 and corresponding, homozygous LBP-knockout mice received a standardized burn, where appropriate followed by topical adenoviral injection encoding rat-LBP or control β-gal. Three days postburn, $1x10^5$multiresistant *Pseudomonas aeruginosa* were topically seeded and covered by occlusive dressings. Seventy-two hours later, the skin was harvested for quantitative bacterial counts.

Results:
In vitro, the efficacy of the adenoviral constructs was confirmed by light microscopy, Xgal staining (β-gal expression) and Western blotting (LBP expression) using mouse fibroblasts. *In vivo*, positive Xgal staining in burned mouse skin was achieved indicating successful gene transfer. Bacterial counts on the skin of mice subjected to burn and infection did not differ between LBPko mice and wt controls. Both, in wild-type animals and in LBPko animals, application of the LBP-producing virus resulted in 4,9-fold respectively 44-fold lower bacterial counts compared to the counts in β-gal transfected mice.

Conclusion:
In a standardized burn wound model, restoring LBP in LBP-knockout animals as well as overexpression of LBP in wild type mice, resulted in improved local immunity. LBP thus plays an important role in topical innate immune response to Gram-negative bacteria. Adenoviral over-expression of LBP has a protective effect against local wound infection and may therefore be considered as a future therapeutic approach for Gram-negative burn wound infections.

Introduction:

Burns are the fourth leading cause of unintentional injury death in the US and account for 3% of all injury deaths (1). In 2010, 450,000 medical treatments resulting from burns led to 45,000 hospitalizations and on average 5,000 extensive surgical reconstructions and interventions (2). With skin the first line of defense, being damaged, the host depends on its innate immune system to rapidly respond to invading organisms and initiate further mechanisms of defense (3-6). Subsequent wound infection, especially with Gram-negative bacteria, leads to high morbidity and mortality (7, 8). Various authors describe a death rate up to 75%, directly or indirectly related to infections after burns (5, 9, 10). Costs following thermal injuries and infections are substantial and patients often suffer from time-consuming reconstructive and rehabilitative procedures (8).

Lipopolysaccharide binding protein (LBP), a 58-60.5 kDa acute-phase protein (11), binds with high affinity ($K_d \approx 10^{-9}$M) and specificity to the lipid A portion of bacterial lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria (12). Formerly this protein was thought to be solely produced in the liver, but we have previously shown that expression is also inducible at extrahepatic sites including wounds (13). LBP enhances binding of LPS to membrane-bound CD14 (14), a GPI-anchored protein without transmembrane components. The Toll-like receptor family transmits the LBP-mediated LPS-CD14 signal to intracellular pathways (12, 15, 16). LBP also functions as an opsonin (17). Furthermore, it greatly enhances the bacterial killing ability of neutrophil-derived bactericidal permeability increasing protein (18). Previously, we have demonstrated an inverse relationship between LBP expression levels in burn wounds and the number of bacterial colonies growing in the wound (19), suggesting that local LBP aided wound defenses against infection.

The studies reported here were performed to further test the hypothesis that local LBP expression plays an integral role in innate immune response towards an infection with living, Gram-negative, multidrugresistant *Pseudomonas aeruginosa* bacteria. This bacterium was found on the skin of a burn victim treated at the hospital's intensive care unit, was isolated and subsequently used in our experiments. To study the role of LBP, LBP-knockout mice were available. To confirm a role for LPB we furthermore constructed an adenoviral gene delivery system that encoded rat LBP. With this construct we determined the effect of restoring LBP expression in burn wounds in LBP-knockout mice and the effects of over-expressing LBP in the burn wounds of wild type mice.

Materials and Methods

Animals

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LBP knockout mice were a gift from Dr. Douglas Golenbock (Boston University School of Medicine) (20). The animals had been backcrossed at least 12 times into their C57BL/6 background prior to use and were previously shown to be homozygous. All experiments were performed in accordance with National Institute of Health guidelines for the use of laboratory animals and approval was obtained from the University of Michigan Animal Care and Use Committee. Mice were kept in specific pathogen-free environment under a 12-hour light cycle, and fed rodent chow and water *ad libidum* in micro-isolator containments. For all our experiments, we used 12-14 weeks old, female, age/weight-matched mice. C57BL/6 wild-type animals were purchased from Harlan (Harlan, Indianapolis, IN), and allowed at least 5 days for acclimatization prior to use in experiments.

Bacteria

A silver sulfadiazine-resistant strain of *Pseudomonas aeruginosa* (lot-no. 26-2844) was previously isolated from a human burn patient at University of Michigan burn ICU (21). This bacterium was transferred into 10 ml of trypticase soy broth (Becton Dickinson, Franklin Lakes, NJ) and shaken at 275 rpm overnight at 37° C. The next day, an aliquot of bacteria was mixed with 10 ml of broth and shaken for another 2.5 hours for bacteria to reach semi-log growth phase. After spinning and washing with chilled phosphate buffered saline (PBS, pH 7.3), a suspension of $1x10^5$ bacteria per 100µl in PBS was made.

Adenoviral constructs

Ad-LBP:

Following the methods described by Aoki et al. (22), the University of Michigan Gene Vector Core produced an Ad5 adenovirus with a dl309 backbone containing the information for rat-LBP. Briefly, the secretory tag sequence of the plasminogen activator inhibitor type 1 gene was inserted one amino acid upstream of the histidine tag from the previously constructed transfer vector pBlueBacHis2c, using polymerase chain reaction (PCR).The PCR product was then cut with Mfe/HindIII and subsequently cloned into an adenoviral shuttle vector (pACCMVplmpl-SSP containing a CMV promoter). Next, a Xho/HindIII segment of the vector was replaced with a Xho/HindIII fragment (2.45kb) of rat LBP gene from pBlueBacHis2c(15) *(see Figure 1a).*

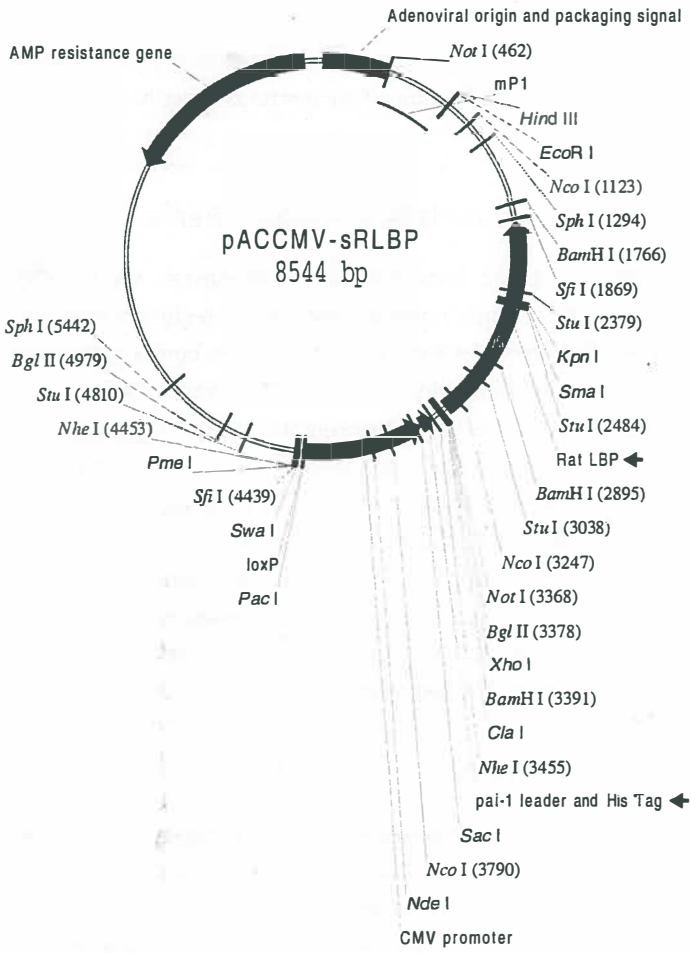

Figure 1a: Schematic representation of the Ad5-adenoviral construct encoding rat LBP used for gene therapy.

The dl309 backbone containing adenovirus (referred to as AdLBP) expresses rat-LBP (upper arrow) under CMV promoter control, and a HIS-tag (lower arrow), and was produced by the University of Michigan Vector Core.

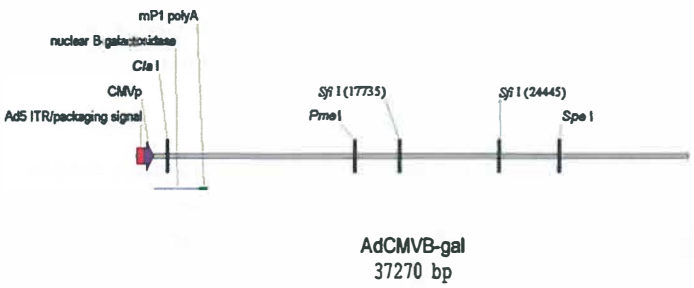

Figure 1b: Control Ad5-adenoviral construct expressing β-galactosidase.

This virus is used as a control adenoviral vector and expresses β-galactosidase (Adβ-gal). When successfully transfected into cells, b-gal activity can be detected as blue when counter-stained with Xgal staining. The construct was made of a similar Ad5-virus with a dl309 backbone as the LBP-producing virus.

Ad-βgal:
The control adenovirus construct expressing β-galactosidase, was made by the University of Michigan Gene Vector Core as well, according to the methods described by Gerard and Meidell (23) *(see Figure 1b)*.

2

In vitro adenoviral gene transfection experiments

NIH/3T3 mouse fibroblasts were purchased from ATCC (ATCC, Manassas, VA, No: CRL-1658) and grown in Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose, and10% bovine calf serum, at 37° C. The adenoviral transfection medium was identical to the growth medium with the exception of the use of 3% bovine calf serum to increase the uptake of the virus. For the *in vitro* transfection experiments, multiplicities of infections (MOI) of 10, 100, 1,000 and 10,000 were used. Adenoviral transfection was performed in 12 well Costar® plates. $5x10^5$ cells/well were seeded into the wells, allowed to rest for 24 hrs, then incubated with virus of the desired MOI for 2 days in transfection medium. The β-gal-construct was used to confirm successful transfection (*see Xgal staining, Figure 2*), using light microscopy evaluation. After 2 days of virus incubation, the supernatants were harvested and the cells were lysed and subjected to Western blotting (*Figure 2)*.

Xgal expression analysis in cell cultures

NIH/3T3 fibroblasts were harvested with Trypsin-EDTA (GIBCO/Invitrogen Life Sciences, Carlsbad, CA), spun, washed and mixed with Xgal fixation buffer (2.7 ml of 37% formaldehyde (Fisher Scientific, Fair Lawn, NJ), 0.4 ml 25% glutaraldehyde (Fisher Scientific, Fair Lawn, NJ), filled to 50 ml with PBS (pH 7.3)) until a total volume of 50 ml was reached for 5 min on ice, followed by rinsing with PBS three times. The resulting cell pellet was

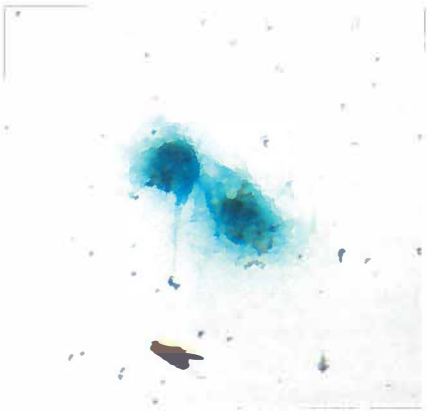

Figure 2: Brightfield microscopic view of two representative 3T3-NIH mouse fibroblasts showing positive Xgal staining.

An example of two viable NIH-3T3 mouse fibroblasts in culture, after successfully being transfected with the Adβ-gal virus 2 days following adenoviral transfection. By Xgal staining these cells appear blue when β-galactosidase is being produced. The multiplicity of infection (MOI) was 100, the picture is taken at 400x magnification.

mixed with Xgal staining solution (1.0 ml X-gal solution stock (25 mg/ml) 50 µl $MgCl_2$, 41mg potassium-ferricyanide, 53mg potassium-ferrocyanide, mixed with PBS (pH 7.3) to a total volume of 25 ml) and placed in an incubator at 37° C overnight. Next, cells were washed, the cell solution was then mounted onto object slides and subjected to microscopic analysis.

Xgal staining of mouse tissue skin samples

OCT frozen skin samples were cut in 5-micron sections, transferred onto standard slides and processed by soaking in 10% formaldehyde (Fisher Scientific) overnight, followed by dehydration in 70% ethanol, embedding in paraffin and subsequent Xgal staining, followed by counterstaining with Eosin and Nuclear Fast Red. Samples were soaked in fixation buffer (4% formaldehyde, 25% glutaraldehyde (Fisher Scientific), 100 µl 10% Tween-20, Lac-Z-rinse (50 ml PBS-Tween (pH 7.3), 1 m $MgCl_2$, 100 µl Tween-20, 448ml water) (InvivoGen)) for 5 minutes at room temperature. The slides were rinsed twice with 1xPBS-Tween and placed into Lac-Z-rinse for 10 minutes. Subsequently, 200µl of staining solution (200µl 0.4 M K-ferricyanide solution (325 mg potassium ferricyanide powder (K_3FeCN_6) in 5 ml 1xPBS (pH 7.3) and 200µl of 0.4-M K-ferrocyanide (potassium ferrocyanide ($K_4Fe(CN)_6*3H_20$) in 5ml 1xPBS (pH 7.3) with 10 mg X-gal 0.25ml (40mg/ml)) was added onto the specimens and incubated overnight (12-18 hrs). The next morning, the specimens were rinsed with 1xPBS-Tween, soaked for 5 minutes in PBS-Tween and counter-stained with Nuclear Fast Red and Eosin).

Western blot analysis of LBP production in cell cultures

Cells transfected *in vitro* by the adenoviral constructs and their corresponding supernatants were harvested, and the cells were mechanically lysed. Cell extracts and supernatants were separated by SDS-PAGE using a 10-12.5% gel under reducing condition using the method of Laemmli (24). The protein transfer was performed electrophoretically by the method of Towbin et al. (25) to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH). The membrane was probed with either mouse monoclonal anti-HIS antibody (Invitrogen Life Sciences, Carlsbad, CA; No. R93025) or mouse monoclonal anti-Xpress antibody (Invitrogen, No. R91025), followed by a horseradish peroxidase-linked goat anti-mouse IgG antibody (Schleicher and Schuell). Detection was carried out with the Western Lightning (Western Lightning kit, PerkinElmer Life Sciences, Boston, MA) blotting kit according to the manufacturer's guidelines, normalized to GAPDH as a housekeeping gene. The protein concentration was standardized by means of a Bio-Rad kit (Bio-Rad, Hercules, CA).

Mouse burn model

For each experiment, eight female C57BL/6 mice (Harlan, Indianapolis, IN) and eight age and weight–matched female LBP knockout mice were clipped at their dorsum under general anesthesia. Anesthesia was given by intraperitoneally (ip) injection of 60-80 mg/kg Ketaset® (Ketamine HCl, 100mg/ml, Fort Dodge, Fort Dodge, IO) and 15 mg/kg AnaSed® (Xylazine 20mg/ml, Lloyd Laboratories, Shenandoah, IO). The animals were then depilated with a depilatory lotion (Nair®, Carter Products, New York, NY) and placed in a mold exposing 25% of their dorsum in a hot water bath by partially submerging (60° C for 10s), to create a partial thickness burn wound. The burn wound size was determined by the Meeh formula (26). Immediately postburn the skin was dried and animals resuscitated with ip saline. All animals received an intra-/ subdermal injection of either the b-gal (control group) or rat-LBP-expressing adenovirus per group. We injected 10^{10} plaque-forming units (pfu) in 100 μl solution of both constructs under sterile conditions, pfus were determined by plaque assay prior to the experiment. For postoperative analgesia, buprenorphine was injected subcutaneously every 6-8 hours for 2 days (Buprenex®, Reckitt & Colman, Richmond, VA). The adenoviral injection site was marked on the skin and covered with a sterile dressing (Telfa® (Non-adherent dressing, Kendall, Mansfield, MA), Tegaderm® (3M Health Care, St.Paul, MN) for occlusive dressing and Flex-Wrap® (Kendall, Mansfield, MA) to prevent animals from removing their dressings). Seventy-two hours post-burn, we performed a dressing-change and applied 10^5Silvadene®-resistant *Pseudomonas aeruginosa* topically onto the site of burn and viral treatment, followed by another occlusive dressing. Seventy-two hours after wound challenge with bacteria, the animals were euthanized for tissue harvest.

Pseudomonas colony analysis in skin samples

Skin was harvested under sterile conditions, weighed and homogenized in saline on ice. Homogenates were plated in serial dilutions in triplicates (10^2, 10^4, 10^6) on 5% sheep-blood agar plates. The following day, the *Pseudomonas* colonies were counted in a blinded fashion and standardized per g/skin, presented as calculated mean of plates, done in triplicates. *Pseudomonas* growth was revealed by morphology, odor and colony aspect.

Statistical analysis

Statistical analysis was performed by using StatView software (SAS Institute, Cary, NC). Data shown express mean value ± 1 SE (standard error), comparison was performed using two-tailed Student's *t* test.

Results

Adenoviral transfection in fibroblasts

In order to proof the efficacy of transfection and protein production of the adenoviral constructs, both were tested in mouse fibroblasts. In our *in vitro* experiments, light-microscope evaluations revealed an average β-gal transfection rate (blue staining of fibroblasts) of approximately 30% (data not shown). As demonstrated in *Figure 2*, positive β-gal transfection turned viable cells blue. As for the LBP-producing virus, Western blot analysis confirmed successful expression of the protein LBP (*Figure 3*) which showed up at the expected size of LBP (58-60 kDa). A previously produced LBP was run in the same gel with the fibroblast supernatants to confirm LBP production. In order to find a virus concentration – production response relation, different MOIs were tested and LBP expression was found to follow increasing MOI as shown in *Figure 3*.

Adenoviral transfection in vivo

Based on the successful *in vitro* transfection of both adenovirus constructs, further *in vivo* testing was done by applying them to the burn wound model. Examination of burn wounds 6 days following burn injury and immediate treatment with adenovirus, demonstrated that the burn wound is capable of expressing the transgene, as seen in *Figure 4*. Positively β-gal transfected intradermal cells showed blue staining, similar to what is seen in culture, in contrast to non-infected cells. Adenoviral encoded protein expression could be seen intra- as well as subdermally.

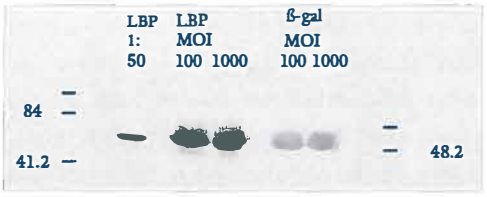

Figure 3: Western blot analysis of proteins derived from lysates of cells transfected with either adenovirus producing rat-LBP or β-gal.

Adenovirus driven LBP protein production was positively identified by detection of a protein band at 58-60 kDa with similar identity as the reference LBP.

Molecular weight ladder left and right enabled identification, left band (LBP 1:50), shows the reference LBP-protein band. The two following dark bands suggest positive protein production at the expected size of 58-60 kDa with MOIs of 100 respectively 1,000. The protein products of the Adβ-gal virus (β-gal MOI 100 and 1,000) acts as a control.

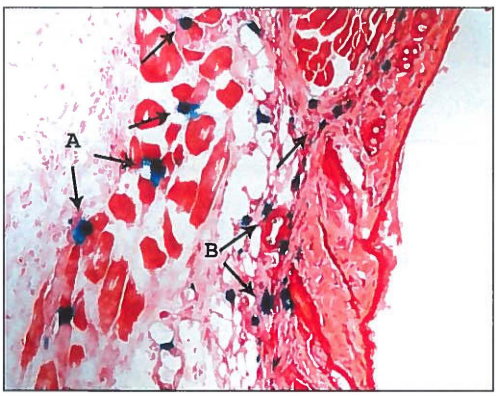

Figure 4: Rodent skin sample 2 days after intradermal adenoviral injection.
Positive transfection is visible intra- and subdermally by blue staining (Xgal stain for β-galactosidase, counterstained with nuclear fast red). The virus concentration administered was 1.10^{10} particle-forming units (pfus) in 100 μl.
Arrows 'A' point to subdermally stained cells, arrows 'B' point to intradermally stained cells (basal layer). The picture is taken at 200x magnification

Burn model and bacterial challenge

After the development of the burn wound model and the proof that the adenoviral constructs expressed either LBP or β-gal, a bacterial challenge study with living, multiresistant *Pseudomonas aeruginosa* was performed. All but one animal (6,3%, n=1/16) survived the experimental course. In order to focus on the effect of these bacterial colonies only, we tried to prevent cross contamination. Therefore, to keep the wound environment standardized, we designed the occlusive dressing as described before.

Three days following burn and infection with the multiresistant *Pseudomonas*, less bacteria were found on the skin of wild-type animals compared to the numbers on the skin LBPko mice, although this was not statistically significant *(Figure 5)*.

In order to test our hypothesis that LBP would be beneficial in clearing bacteria, both adenovirus constructs were injected into the living, regenerating wound ground of the wild type C57BL/6 mice. Another 72 hours after infliction of the defined burn wound followed by infection, the bacterial numbers were compared, as can be seen in *Figure 6*. The numbers were statistically significantly lower on the LBP-transgene skin by 44-fold compared to the numbers on the skin of the β-gal transfected mice.

The same approach was repeated by injecting either viral construct into the skin of LBP-knockouts, followed by standardized infection and quantitative counts (*Figure 7)*. In knockouts where the LBP encoding virus had been injected and thus LBP has been restored, we found that *Pseudomonas* numbers were lowered by 4.9-fold, than the numbers found upon administration of the control virus. This difference was statistically significant ($p < 0.03$).

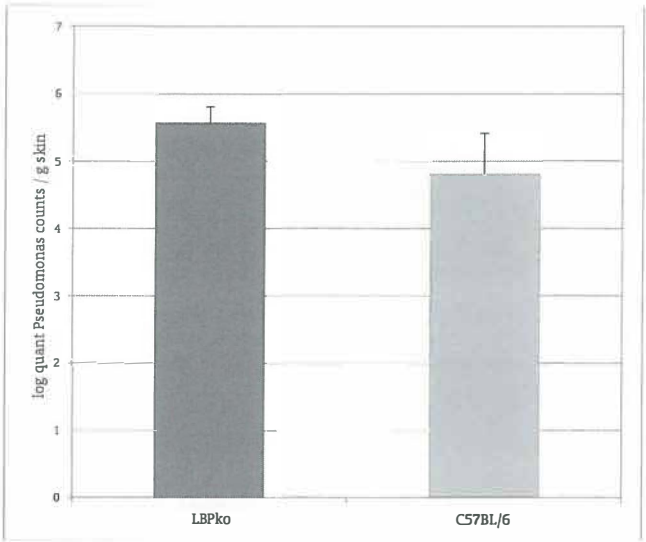

Figure 5: Comparison of *Pseudomonas* bacterial numbers on skin of LBP-knockout animals versus wild types following burn and defined infection.

Three days following burn wound and infection with $1x10^5$ *Pseudomonas aeruginosa* bacteria, quantitative bacterial counts were performed, comparing knockout animals to wild-types. No statistical significance was found using Student's *t*-test, the bacterial numbers were comparable. (n=8 animals / group, data are presented showing +/– 1 SE)

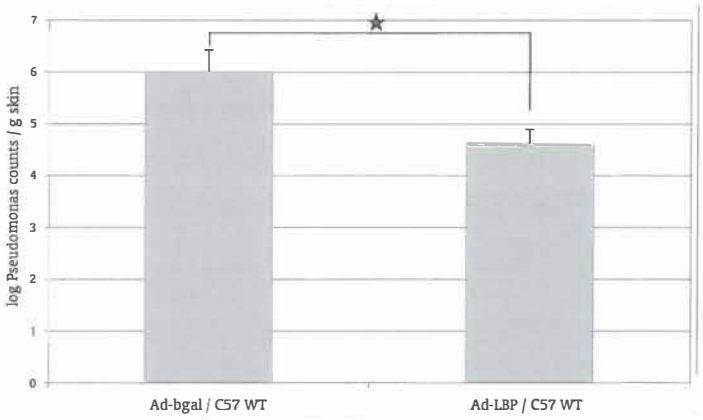

Figure 6: *Pseudomonas* bacterial numbers on wild type mice, comparing Ad-bgal versus Ad-LBP treatment.

Either adenovirus was injected intradermally into wild type mice (C57 WT) immediately after the burn was covered by sterile dressing, three days later the animals were infected by topical application of $1x10^5$ bacteria. Another three days later, quantitative bacterial counts of treated and infected dermis were performed. The Ad-LBP virus reduced the *Pseudomonas* numbers by 44-fold on wild type skin.

Bars represent the mean values of 8 mice, with +/– 1 SE. Asterisk sign indicates significance ($p<0.03$)

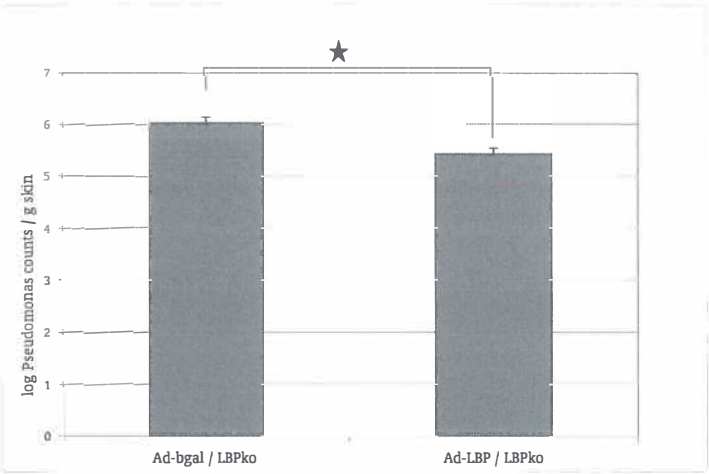

Figure 7: *Pseudomonas* bacterial numbers on LBP-knockout mouse skin, comparing Ad-bgal treatment versus Ad-LBP treatment.

After inflicting the burn wound, the LBP-knockout mice were injected with adenoviral construct and covered by sterile wound dressings for 72 hours, then got infected by $1x10^5$ Pseudomonas aeruginosa bacteria, covered again and another 72 hours later the skin was harvested.

'Restoring' LBP by means of the adenoviral construct (Ad-LBP / LBPko) led to a statistically significant reduction of bacterial numbers by 4.9-fold, when compared to the control virus. Asterisk sign indicates significance with $p<0.03$ (n=8 animals /group, +/- 1 SE)

Discussion

Burn wound infections remain common despite the high standard of care on burn intensive care units. Depending on burn depth and extent, sepsis emerging from wound infection following burns remains a major cause of mortality and morbidity (27), making especially Gram-negative infections troublesome. By establishing a standardized, partial thickness burn wound model in rodents, we could test our hypothesis that local LBP expression plays an important role in the innate immune response towards Gram-negative bacteria. Using wild type and LBP-knockout mice, as well as an adenoviral gene delivery system encoding LBP, we showed that genetic ablation of LBP expression *per se* did not affect bacterial counts in the model used. In addition, we could demonstrate a significant decrease in topical bacterial numbers in mice treated with the LBP-encoding adenovirus, both in wild type and in LBP-knockout mice. This adenoviral over-expression of LBP might be considered as a future therapeutic approach to diminish bacterial load in critical wounds, such as burns.

In Gram-negative bacterial infections as such, lipopolysaccharide-binding protein (LBP) as part of the innate immune system, is considered to play a key role (28-30). The generally accepted dogma states that LPS, as a major component of the outer cell wall of Gram-negative bacteria, binds to LBP, further initiating an immune response, but the importance of LBP in partial thickness burns remains rather unclear (19). Consequently, we developed a burn wound model, enabling us to focus on LBP and innate immunity, by means of an LBP-knockout mouse colony, for which we adapted exposure time and mold design previously found useful in our roughly 10-times bigger rat burn-wound model (19).

We postulated that LBP is an important component of local wound defense against bacterial infection in a rodent burn wound model, and that local over-expression of LBP within the wound site may be therapeutically advantageous. With regard to our LBP-knockout mouse colony being unable to produce LBP, complex immunologic mechanisms involving Gram-negative infections are expected to be profoundly different when compared to wild-type animals. In our case however, we did not observe a deleterious effect on bacterial clearance of knockout animals lacking LBP. It is accepted, that LBP facilitates the binding of LPS to CD14, a membrane-bound anchor protein without transmembrane components found on monocytes, macrophages and neutrophils. Alternatively, LBP delivers LPS to soluble CD14 (sCD14) to form LPS-sCD14 complexes that may activate a much larger variety of cell types, including CD14-negative cells such as endothelial cells (31, 32). The animals used in our experiments, however, were incapable of producing a functional LBP. Therefore, another way of responding to an defined LPS challenge by, as

in our case *Pseudomonas*, possibly existed, since we did not find differences in bacterial numbers on LBP-competent (wt) and incompetent (LBPko) mice. Although LBP was shown not to be required *in vivo* to clear LPS (delivered through Gram-negative infections), in an animal study with LBP-deficient mice (33), it was shown to be necessary for the rapid induction of an inflammatory response by small amounts of LPS or Gram-negative bacteria. LBP and CD14 together represent the major pathway by which cells recognize low concentrations of LPS (34), presenting the LPS-LBP binding signal to members of the Toll-like receptor family (TLRs), mediating it to intracellular compartments (35, 36). Specifically, both TLR2 and TLR4 are highly expressed by cells in response to LPS-exposition, such as macrophages and monocytes (15, 37, 38), but only TLR4 is accepted as an LPS signaling receptor (39). Many downstream activators are triggered by the LPS signaling in monocytes, for instance rapid tyrosine phosphorylation of proteins, such as the *src* tyrosine kinase family members (40). LBP-LPS binding stimulates macrophages and monocytes to produce inflammatory proteins such as TNFα, IL-6 or IL-1 (40, 41). Since no functioning LBP-protein was produced in the LBPko mice used in our experiments, one can speculate, that there were possible compensatory mechanisms enabling burned and infected LBPko mice to demonstrate the same bacterial clearance as their corresponding wild types.

The knockout animal concept is *per se* an investigatory tool, and its resulting information can be expanded by restoring the gene which is initially knocked-out, for instance using gene-therapy. Currently available vectors for introduction of transgene encoding information include retroviruses, liposomes, plasmids, gene-gun delivery, and adenoviruses (42). We chose adenoviruses because they are quite easy to use, allow transient transfection of CAR (coxsackie adenovirus receptor)-positive cells (43), are not integrated into the host genome, and can be easily administered locally *in vivo*, in our case intradermally. Usually an unpredictable, certain level of locally induced inflammation follows adenoviral administration and might be considered as a disadvantage. In our experiments, the positive effect of transgene expression was notably higher than the theoretical, negative side-effect caused by adenovirus-induced inflammation, as seen by the reduction of bacteria demonstrated in *Fig.6* and *Fig.7*. In our experiments, we used an E1 and E3 deleted adenoviral (Ad5) construct. Literature reveals that administration of lower dosages than those we had injected (8.10^7 and 8.10^9 plaque-forming units (pfu) rather than 10^{10} pfus as used in our case) are sufficient to generate an immune reaction by recruiting T-and B-cells, as well as macrophages at day 3 post-transfection, being scored as mild to moderate (44). Possibly, the described recruitment of immune-competent cells by an adenovirus is not of disadvantage, but actually beneficial in terms of clearance of topical bacteria as in our case. Using the LBP-producing virus in wild type mice, reduced the bacterial numbers by 44-fold and in knockouts by 4.9-fold, where LBP was restored by the virus.

Conclusion

In a standardized rodent, partial thickness burn wound model we demonstrated that enhancing LBP expression at the site of LPS-challenge represented by living, multiresistant *Pseudomonas aeruginosa*, led to decreased bacterial colony growth, both in wild type and LBP knockout mice. Based on these observations we conclude that LBP plays an important role in innate immune responses against Gram-negative bacterial infection, and that stimulation of LBP expression can have a protective effect against wound infection. In our hands, adenoviral gene therapy-driven production of LBP in a partial thickness burn wound model was feasible. Further investigations are needed to understand the complex immunologic processes affected by LBP-LPS-interaction more clearly.

References:

1. Liao CC, Rossignol AM. Landmarks in burn prevention. *Burns* 2000;26:422-434.
2. National Burn Registry Annual Report 2010. Available at: http://www.ameriburn.org/2010NBR AnnualReport.pdf.
3. McManus WF, Goodwin CW, Mason AD, Jr., et al. Burn wound infection. *J Trauma* 1981;21:753-756.
4. Wolf SE, Sterling JP, Hunt JL, et al. The year in burns 2010. *Burns* 2011;37:1275-1287.
5. Kasten KR, Makley AT, Kagan RJ. Update on the critical care management of severe burns. *J Intensive Care Med* 2011;26:223-236.
6. Rafla K, Tredget EE. Infection control in the burn unit. *Burns* 2011;37:5-15.
7. Bloemsma GC, Dokter J, Boxma H, et al. Mortality and causes of death in a burn centre. *Burns* 2008;34:1103-1107.
8. Azzopardi EA, Azzopardi SM, Boyce DE, et al. Emerging gram-negative infections in burn wounds. *J Burn Care Res* 2011;32:570-576.
9. Revathi G, Puri J, Jain BK. Bacteriology of burns. *Burns* 1998;24:347-349.
10. Mann EA, Baun MM, Meininger JC, et al. Comparison of mortality associated with sepsis in the burn, trauma, and general intensive care unit patient: a systematic review of the literature. *Shock* 2012;37:4-16.
11. Tobias PS, Soldau K, Ulevitch RJ. Isolation of a lipopolysaccharide-binding acute phase reactant from rabbit serum. *J Exp Med* 1986;164:777-793.
12. Bosshart H, Heinzelmann M. Targeting bacterial endotoxin: two sides of a coin. *Ann N Y Acad Sci* 2007;1096:1-17.
13. Su GL, Freeswick PD, Geller DA, et al. Molecular cloning, characterization, and tissue distribution of rat lipopolysaccharide binding protein. Evidence for extrahepatic expression. *J Immunol* 1994;153:743-752.
14. Wright SD, Ramos RA, Tobias PS, et al. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* 1990;249:1431-1433.
15. Su GL, Klein RD, Aminlari A, et al. Kupffer cell activation by lipopolysaccharide in rats: role for lipopolysaccharide binding protein and toll-like receptor 4. *Hepatology* 2000;31:932-936.
16. Knapp S, von Aulock S, Leendertse M, et al. Lipoteichoic acid-induced lung inflammation depends on TLR2 and the concerted action of TLR4 and the platelet-activating factor receptor. *J Immunol* 2008;180:3478-3484.
17. Tobias PS, Soldau K, Ulevitch RJ. Identification of a lipid A binding site in the acute phase reactant lipopolysaccharide binding protein. *J Biol Chem* 1989;264:10867-10871.
18. Iovine N, Eastvold J, Elsbach P, et al. The carboxyl-terminal domain of closely related endotoxin-binding proteins determines the target of protein-lipopolysaccharide complexes. *J Biol Chem* 2002;277:7970-7978.
19. Klein RD, Su GL, Aminlari A, et al. Skin lipopolysaccharide-binding protein and IL-1beta production after thermal injury. *J Burn Care Rehabil* 2000;21:345-352.

20. Wurfel MM, Monks BG, Ingalls RR, et al. Targeted deletion of the lipopolysaccharide (LPS)-binding protein gene leads to profound suppression of LPS responses ex vivo, whereas in vivo responses remain intact. *J Exp Med* 1997;186:2051-2056.
21. Steinstraesser L, Klein RD, Aminlari A, et al. Protegrin-1 enhances bacterial killing in thermally injured skin. *Crit Care Med* 2001;29:1431-1437.
22. Aoki K, Barker C, Danthinne X, et al. Efficient generation of recombinant adenoviral vectors by Cre-lox recombination in vitro. *Mol Med* 1999;5:224-231.
23. Gerard, R.D., Meidell, et al. *DNA Cloning: A Practical Approach.* Oxford: Oxford University Press; 1995.
24. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680-685.
25. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci US A* 1979;76:4350-4354.
26. Gilpin DA. Calculation of a new Meeh constant and experimental determination of burn size. *Burns* 1996;22:607-611.
27. Branski LK, Al-Mousawi A, Rivero H, et al. Emerging infections in burns. *Surg Infect (Larchmt)* 2009;10:389-397.
28. Freudenberg MA, Tchaptchet S, Keck S, et al. Lipopolysaccharide sensing an important factor in the innate immune response to Gram-negative bacterial infections: benefits and hazards of LPS hypersensitivity. *Immunobiology* 2008;213:193-203.
29. Zweigner J, Schumann RR, Weber JR. The role of lipopolysaccharide-binding protein in modulating the innate immune response. *Microbes Infect* 2006;8:946-952.
30. Kitchens RL, Thompson PA. Modulatory effects of sCD14 and LBP on LPS-host cell interactions. *J Endotoxin Res* 2005;11:225-229.
31. Tapping RI, Tobias PS. Cellular binding of soluble CD14 requires lipopolysaccharide (LPS) and LPS-binding protein. *J Biol Chem* 1997;272:23157-23164.
32. Dunzendorfer S, Lee HK, Soldau K, et al. TLR4 is the signaling but not the lipopolysaccharide uptake receptor. *J Immunol* 2004;173:1166-1170.
33. Jack RS, Fan X, Bernheiden M, et al. Lipopolysaccharide-binding protein is required to combat a murine gram-negative bacterial infection. *Nature* 1997;389:742-745.
34. Cunningham SC, Malone DL, Bochicchio GV, et al. Serum lipopolysaccharide-binding protein concentrations in trauma victims. *Surg Infect (Larchmt)* 2006;7:251-261.
35. Blomkalns AL, Stoll LL, Shaheen W, et al. Low level bacterial endotoxin activates two distinct signaling pathways in human peripheral blood mononuclear cells. *J Inflamm (Lond)* 2011;8:4.
36. Tsukamoto H, Fukudome K, Takao S, et al. Lipopolysaccharide-binding protein-mediated Toll-like receptor 4 dimerization enables rapid signal transduction against lipopolysaccharide stimulation on membrane-associated CD14-expressing cells. *Int Immunol* 2010;22:271-280.
37. Hajishengallis G, Martin M, Schifferle RE, et al. Counteracting interactions between lipopolysaccharide molecules with differential activation of toll-like receptors. *Infect Immun* 2002;70:6658-6664.
38. Toshchakov V, Jones BW, Perera PY, et al. TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages. *Nat Immunol* 2002;3:392-398.
39. Raetz CR, Whitfield C. Lipopolysaccharide endotoxins. *Annu Rev Biochem* 2002;71:635-700.

40. Guha M, Mackman N. LPS induction of gene expression in human monocytes. *Cell Signal* 2001;13:85-94.
41. Ren L, Leung WK, Loo TW, et al. Lipopolysaccharide-binding protein down-regulates the expression of interleukin-6 by human gingival fibroblast. *J Periodontal Res* 2005;40:407-416.
42. Khavari PA, Rollman O, Vahlquist A. Cutaneous gene transfer for skin and systemic diseases. *J Intern Med* 2002;252:1-10.
43. Evans ME, Lesnaw JA. Infection control for gene therapy: a busy physician's primer. *Clin Infect Dis* 2002;35:597-605.
44. Harvey BG, Worgall S, Ely S, et al. Cellular immune responses of healthy individuals to intradermal administration of an E1-E3- adenovirus gene transfer vector. *Hum Gene Ther* 1999;10:2823-2837.

Chapter 3

Alterations in intradermal gene expression profiles by lipopolysaccharide binding protein (LBP) in a rodent burn model

3

Abstract

Objective

Lipopolysaccharide binding protein (LBP) is important in fighting Gram-negative bacterial infection. In previous experiments, topical *Pseudomonas aeruginosa* colonies were found to be reduced upon overexpression of LBP in a partial thickness burn model in small rodents. The current objective was to investigate intradermal and systemic immunological alterations in response to burn infliction in the presence or absence of LBP, using an LBP-knockout (LBPko) mouse colony compared to their wild-type (wt) controls.

Materials Methods

Three age/weight matched female C57BL/6 and 3 LBP-knockout mice sustained a 25% total body surface partial thickness burn. Six hours later the burn skin-RNA was extracted and subjected to gene expression profiling. The experiment was repeated including six animals in either group with their skin harvested 24 hours post burn and analyzed for IL-10, TNFa, GRO-1 and IL-6, using real time RT-PCR. Furthermore, differential leukocyte analysis of peripheral blood obtained from each animal was performed.

Results

Gene chip analysis revealed profound changes in intradermal gene expression patterns of one chemokine and several inflammatory cytokines in LBPko versus wild-type animals at 6 hours following burn. The highest expression level was found for the CXC-chemokine growth-related oncogene 1 (GRO-1), with a more than 52-fold up-regulation in knockouts compared to controls. Total white blood cells, neutrophils and lymphocytes were increased in LBP-knockouts at 24 hours after the burn, when compared to an uninjured state. In contrast, only the numbers of peripheral monocytes in wild-types were significantly increased at 24 hours.

Conclusion

With regard to intradermal gene expression, LBP-knockout and wild-type mice demonstrated pronounced differences when subjected to a standardized, partial thickness burn wound. The GRO-1 gene revealed a more than 52-times higher expression level in knockouts compared to control. Profound differences between the animal groups were also noted at 24 hours after the injury in both peripheral leukocyte composition and quantitative numbers. LBPko mice reacted with neutrocytosis, lymphocytosis and increased total white blood cells, whereas wild types showed monocytosis at 24 hours after trauma. From this it can be concluded that knocking out LBP has an effect on the rodent's immune system, both locally and systemically when challenged by a burn. Whether LBP itself or possible compensatory mechanisms are responsible for the altered immunity encountered, needs further investigation.

Introduction

Burn wounds still represent a challenge for the treating physician, since infected burn wounds remain a source of high morbidity and mortality (1). In the US, the lethality arising from burns in males is roughly 4% whereas in females it is 5.5% (2). In 2010, 450,000 medical treatments resulting from burns led to 45,000 hospitalizations and on average 5,000 extensive surgical reconstructions and interventions (3). Because the skin as a defensive barrier is damaged as a result of scald injury, the body needs to respond to invading bacteria by activating the innate immune system (4, 5). Consequently, inflammatory reactions lead to cellular activation and migration. Effector cells such as leukocytes are attracted towards the site of injury and contribute to the outcome of the initiated immune response. Among infections, Gram-negative bacteria are challenging to eradicate (6, 7), since they favor wound fluids and moist dressings, conditions commonly found in burn care treatment. 'Biofilms' are formed, which surround the bacteria with a sticking, mechanically protective protein barrier. Despite all efforts and modern methods of treatment, the death toll emerging from Gram-negative sepsis remains high.

Previously, we have reported on lipopolysaccharide-binding protein (LBP) being produced in partial thickness burn wounds of rodents (8). Lipopolysaccharide binding protein, a 58-60.5 kD acute-phase protein (9), binds with high affinity and specificity to the lipid A portion of bacterial lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria (10). LBP is capable of inducing the production of inflammatory cytokines, recruiting immunocompetent cells, containing invading organisms, and clearing noxious substances from wounds. Our group has demonstrated extrahepatic, cutaneous LBP-production, while formerly it was solely thought to be produced in the liver (11). LBP is thought to play an important role in Gram-negative immune responses.

Wound healing is an interactive, dynamic process orchestrated by soluble mediators, extracellular matrix components, as well as resident cells such as fibroblasts, keratinocytes, endothelial cells, nerve cells, and infiltrating leukocytes. It is now well accepted that chemokines, low molecular weight, secreted proteins, contribute to regulating leukocyte influx, and orchestrate the body's response to an immunogenic challenge involving leukocyte trafficking and migration (12-14).

Using a standardized, infected partial thickness burn model in small rodents, we previously have conducted experiments aimed at deciphering the role of LBP in topical Gram-negative bacterial clearance. Since the effects of LBP-LPS interaction under the conditions created in a partial thickness burn wound remain unclear, we were in the current study

interested in the intradermal and systemic immunological processes triggered by the absence or presence of LBP. For this, we first performed intradermal gene chip analysis of partially burned skin 6 hours after burn infliction, possibly revealing differences in gene expression levels in LBP-knockout mice compared to wild-types without infection. In addition, intradermal mRNA levels of pro- and anti-inflammatory cytokines were analyzed at 24 hours after the burn. Finally, we investigated possible differences in peripheral leukocyte subset cell counts in both animal groups in an uninjured state and at 24 hours post-burn.

Methods

LBPko mouse strain

LBP knockout mice were received from Douglas Golenbock (Boston University School of Medicine) (15). These animals had been backcrossed at least 12 times into the background C57BL/6 strain prior to use. All experiments were performed in accordance with the regulations of the National Institute of Health guidelines for the use of laboratory animals, and approval was obtained from the University of Michigan Animal Care and Use Committee. All mice were kept in specific pathogen-free environment under a 12-hour light cycle, fed with rodent chow and water *ad libidum* in micro-isolator containments. For all our experiments, we used female 12-14 weeks old, age/weight-matched LBP-knockout and wild-type mice. C57BL/6 wild-type animals were purchased from Harlan (Harlan, Indianapolis, IN) and allowed at least 5 days for acclimatization prior to use in experiments.

Mouse burn model and animal groups

Animals for gene chip analysis

Three female C57BL/6 wt mice and 3 age/weight–matched female LBPko mice were clipped at their dorsum under general anesthesia (using Ketaset® (Ketamine HCl, 100mg/ml, Fort Dodge, Fort Dodge, IO) and AnaSed® (Xylazine 20mg/ml, Lloyd Laboratories, Shenandoah, IO)) applied by intraperitoneal (ip) injection. The animals were then depilated with a depilatory lotion (Nair®, Carter Products, New York, NY) and placed in a mold exposing 25% of their dorsum in a hot water bath (60° Celsius for 10s) by partial submersion, creating a partial thickness burn wound, type IIB. The wound size was determined and calculated following the Meeh formula (16). Immediately postburn, the skin was dried and animals resuscitated with ip saline. Six hours after burn, the animals were euthanized by an overdose of ip Pentobarbital (Pentobarbital Sodium 50mg/ml, Abbott Laboratories, North Chicago, IL) and the skin harvested under sterile conditions to be subjected to intradermal gene expression analysis.

Animals for real time RT-PCR analysis at 24 hours postburn

Six female C57BL/6 mice (Harlan) and six matched in-house bred female LBPko mice were treated according to the burn model described above. Following the burn, weight-controlled Buprenorphine (Buprenex®, Buprenorphine HCl, Reckitt & Colman, Richmond, VA) was injected subcutaneously well away from the burn site every 6-8 hours. Twenty-four hours later, the animals were sacrificed by an overdose of ip Pentobarbital (Pentobarbital Sodium 50mg/ml, Abbott Laboratories, North Chicago, IL). At the time of sacrifice, skin samples were harvested under sterile conditions for real time reverse-transcription polymerase chain-reaction (RT-PCR) and whole blood was taken by cannulating the inferior

caval vein. A small portion of the whole blood of each animal was mixed with EDTA for use in the Hemavet® (Drew Scientific Inc., Dallas, TX) hematology analyzing system. The remaining volume of the drawn blood was centrifuged at 5,000 g for 5 minutes at 4° Celsius, to separate cells from supernatant, the serum was then snap frozen in -80° Celsius for later analysis.

Tissue samples for gene expression

Mouse skin of the partial thickness burn to be subjected to either gene chip analysis or RT-PCR analysis was harvested including the underlying *Panniculus carnosus* and care was taken not to harvest any muscle tissue. Immediately after weighing, the tissue was snap frozen in liquid nitrogen and stored at -80° Celsius until extraction of RNA.

RNA extraction, purification and preparation for *Affimetrix*® gene chip analysis

RNA isolation

All tissue samples were homogenized without being thawed in the presence of TRIzol® reagent (Life Technologies Inc., Gaithersburg, MD) and total cellular RNA was purified according to the manufacturer's guidelines (Affimetrix®, Santa Clara, CA). Thereafter, RNA samples for gene chip analysis were further purified using acid phenol extraction, followed by the protocol for RNeasy® preparation of spin columns (Qiagen, Valencia, Ca). The quality of the extracted RNA was assessed performing a 1% ethidium-bromide agarose gel electrophoresis to determine intact 18S and 28S bands, and confirmed intact RNA in all samples.

cRNA Synthesis, Gene Array analysis, Gene expression profiling and Statistics

For the purpose of this study, gene array analysis was performed in collaboration with the University of Michigan NIDDK Biotechnology Center (supported by NIH grant DK58771). Skin samples were taken at the site of burn, and gene expression levels analyzed in relation to the gene expression of uninjured lung tissue according to the protocols of the NIDDK core facility and then compared to the other group. The preparation of cRNA, as well as hybridization, scanning and finally image analysis followed the manufacturer's protocols and previous publications (17, 18). The arrays were scanned using the GeneArray scanner (Affymetrix®). Image analysis was performed with GeneChip software (Affymetrix®).

In short, commercially available high-density oligonucleotide microarrays (version MG_U74Av2 Affymetrix chip, Affymetrix®, Santa Clara, CA) were used, capable of analyzing 36,000 genes and expressed sequence tags (ESTs) at once. The protocol was set up by the University of Michigan Computational Medicine and Bioinformatics, as well as the Department of Pathology. There were 20 pairs of features (probe-pairs) on the chip for each transcript (probe-set), 20 of which were designed to be complementary to a specific sequence (perfect match, PM), another 20 were identical except for an altered central base (mismatch, MM). The mismatch probes were subtracted from the perfect match values and averaged the middle 50% of the differences as the expression measure for that probe. A quantile normalization procedure was used to adjust for differences in the probe intensity distribution across different chips. A monotone linear spline to each chip was applied that mapped quantiles 0.01 up to 0.99 (in increments of 0.01) exactly to the corresponding quantiles of the standard. Then, the transform log[100 + max(X + 100;0)] was applied to the data from each chip (19). The average intensity for each probe-set was computed as the mean of the PM-MM differences, after trimming away the 25% highest and lowest differences. For comparison of 2 chips, a two-sided Wilcoxon signed rank test was applied to the PM-MM difference after normalization for statistical analysis.

GRO-1 / KC / CXCl1 primer-design

According to the results of the gene chip analysis, the LocusLink and UniGene databanks (*LocusLink No.*14825, *UniGene No.*21013) were used to confirm and identify the gene locus of interest, which has shown the highest gene expression in the gene chip analysis described above. The nucleotide messenger RNA sequence (NCBI nucleotide database: NM_008176, *Mus Musculus* chemokine (C-X-C motif) ligand 1 mRNA (CXCL1, also known as: growth-related oncogene-1 (GRO-1), also known as: Keratinocyte-derived cytokine (KC)),) consisted of 884 base pairs out of which PCR primers were designed for use in real time RT-PCR. Forward (5' sequence: **CAA TGA GCT GCG CTG TCA GTG**, sense) primer and reverse (3' sequence: **AAG CCT CGC GAC CAT TCT TGA**, antisense) primer resulted in a 139 base pair PCR product. To confirm successful primer design, we back-sequenced the resulting PCR product (at University of Michigan, Biomedical Research Core Facility, DNA Sequencing Core) and found a 97% consistency with our original design.

SmartCycler® real time RT-PCR

Cytokine mRNA levels were analyzed in 6 animals of each group 24 hrs postburn to compare LBPko versus C57BL/6 wt mice to be subjected to RT-PCR. Total RNA was isolated with the TRIzol reagent per manufacturer's instructions (Invitrogen, Carlsbad, CA). Reverse transcription was carried out using the high-capacity cDNA reverse transcription

kit (Applied Biosystems, Foster City, CA). Real-time PCR was carried out using the IQ SYBR Green Supermix and the SmartCycler® (Cepheid, Sunnyvale, CA). The GRO1/KC/CXCL1, TNFa, IL-10 and IL-6 real-time PCR included a denaturation step for 120 sec at 95°C and 3 temperature-cycles repeated 45 times (94.0°C for 15s, 64.0°C for 15s, 72.0°C for 40s) followed by a melting curve from 60.0°C to 95.0°C at 0.2°C/second for each sample. The comparative threshold cycle (Ct) was used to determine the relative amount of RNA transcript between experimental samples (20). PCR products were also examined using a 1% agarose ethidium-bromide gel to confirm the correct size of the PCR product for each primer set. Our target gene threshold cycle (Ct) was compared to a housekeeping gene of the same sample (glyceraldehyde-3-phosphate dehydrogenase, GAPDH), results are reported as ratios of target gene Ct / GAPDH Ct (20).

Hemavet blood analysis

Neutrophils are attracted by chemokines such as GRO-1. In order to assess peripheral neutrophil numbers in the blood stream, we analyzed total white blood cell counts, as well as neutrophils, monocytes and lymphocyte counts in mouse whole blood at sacrifice. To be able to compare the animal´s state between uninjured and post-burn, we made a baseline evaluation of peripheral blood leukocytes by subjecting 6 unharmed female LB-Pko and 6 corresponding wild-type mice to whole blood analysis. EDTA anti-coagulated blood samples were used to obtain a complete blood count with a Hemavet® (Drew Scientific Inc., Dallas, TX) hematology analyzing system. Samples were counted within five minutes after the blood was drawn.

Statistics

Analysis was performed using Statview® software (SAS Institute, Cary, NC).
Unless indicated otherwise, data are expressed as the mean ± SE and were compared using a two-tailed Student's *t* test. Statistical significance was assigned at *p*-values <0.05.

Results

Gene chip data

Table 1 shows the outcome of the gene-chip analysis of pooled intradermal RNA of 3 animals per group harvested from LBPko mice and from C57BL/6 wt having sustained a standardized partial thickness burn. Affimetrix® gene chip analysis showed 12,500 genes out of 36,000 analyzed to be altered and that 1,802 had a more than 2-fold difference (increase or decrease) at 6 hours after burn. Out of these, we demonstrate 49 genes related to inflammation.

In the knockout animal group analyzed at 6 hours postburn, the CXC-chemokine GRO-1 (also known as 'KC', 'MIP2' and 'CXCL1', the mouse analogue of human interleukin- 8 (IL-8)) showed an up-regulation of over 52-fold. The differential expression of this gene between the LBPko mice and their wild type controls was found to be highest of all. With regard to inflammatory cytokines, IL-6 was up-regulated 7-fold in LBPko animal skin versus wild-types. IL-1a and IL-10 did not show significance in gene expression between knockouts and wt mice. A family member of tumor necrosis factor alpha (TNFa), TNFa-'protein-2', was found to be down-regulated 2-fold, as were several members of the tumor necrosis factor receptor super-families. The skin antimicrobial peptide defensin β-2 was down-regulated 3-fold in LBPko mice, as was one receptor involved in binding of GRO-1, the CXC-chemokine receptor-2 (CXCR-2). Interleukin-1β was found to be 3-fold down-regulated. Several genes encoding for the heat-shock protein 70 family were found to be markedly up-regulated (*Table 1*).

Quantitative gene expression at 24h after burn

mRNA expression levels of TNFa, GRO-1, IL-10 and IL-6 of skin samples harvested 24 hours after burn infliction were analyzed by real time RT-PCR in both animal groups (*Figure 1*). At this later time point after burn infliction, GRO-1 gene expression levels were found to be higher in wild-type mouse skin than in knockouts. The other cytokines studied showed no differences in their expression when comparing knockouts and wild-types at 24 hours post-burn.

Blood counts

The analysis of total white blood cells (WBC) of uninjured female LBP-knockout animals compared to wild-types, showed no significant differences between the two groups (*Figure 2*), neither did the differential blood cell count.

Table 1:

Affimetrix® gene chip analysis of skins 6 hours after partial thickness burn in control and LBP-knockout mice.

Pooled, extracted skin mRNA was analyzed as described in Material and Methods. The table shows an excerpt of results (49 out of 12,500 regulated genes) of double-sided ('FC either way') Wilcoxon signed rank test, expressing up- or down-regulation of genes, comparing LBPko with C57BL/6 wt animals.

Left column (from top to bottom): genes up-regulated in LBPko mice are expressed in **bold;** down-regulated genes are underlined, **bold** and *italic*; unchanged genes are *italic*.

Examples of unchanged gene expression of pro- and anti-inflammatory cytokines are expressed at the bottom of this table for completion.

Genes coding for Unigene locus	FC either way
GRO1 oncogene	52.9
heat shock 70 kDa protein 4	8.4
heat shock protein, 110 kDa	8.0
interleukin 6	7.0
heat shock protein, 70 kDa 4	3.9
tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	3.7
heat shock protein, 70 kDa 3	3.5
cytokine inducible SH2-containing protein 3, SOCS	3.2
heat shock protein, 70 kDa 1	3.0
programmed cell death 8 (apoptosis inducing factor)	3.0
coxsackievirus and adenovirus receptor	2.9
interleukin 11 receptor, alpha chain 1	2.9
chemokine (C-C) receptor 2	2.7
interleukin 1 receptor, type I	2.6
tumor necrosis factor receptor superfamily, member 1a	2.5
interleukin 10 receptor, beta	2.5
heat shock 70kD protein 5 (glucose-regulated protein, 78kD)	2.5
tumor necrosis factor receptor superfamily, member 19	2.5
heat shock protein, 110 kDa	2.3
heat shock protein, 86 kDa 1	2.3
heat shock 10 kDa protein 1 (chaperonin 10)	2.2
LPS-induced TNF-alpha factor	2.2
chemokine (C-X-C) receptor 4	2.1
tumor necrosis factor, alpha-induced protein 2	2.1
interleukin 1 receptor antagonist	2.1
interleukin 1 receptor, type II	2.1
heat shock protein, 25 kDa	2.2
interleukin 6 receptor, alpha	2.2
tumor necrosis factor receptor superfamily, member 18	2.2
interleukin 3	2.4

heat shock protein, 30 kDa	2.4
fibroblast growth factor binding protein 1	2.5
heat shock protein, 84 kDa 1	2.7
chemokine (C-C) receptor 1, -like 1	2.7
interleukin 12 receptor, beta 2	2.8
tumor necrosis factor receptor superfamily, member 17	2.9
tumor necrosis factor (ligand) superfamily, member 10	3.0
tumor necrosis factor (ligand) superfamily, member 11	3.0
interleukin 3 receptor, alpha chain	3.0
defensin beta 2	3.1
chemokine (C-X-C) receptor 2	3.4
interleukin 1 beta	3.9
CD4 antigen	4.1
chemokine (C motif) XC receptor 1	4.3
interleukin 1 alpha	*1.0*
interleukin 10	*1.0*
interleukin 12a	*1.0*
interleukin 12b	*1.0*

When analyzed at 24 hrs following burn and compared to the 'baseline reading' (i.e., at an uninjured state) (*Figure 3*), the data indicated an almost two-fold increase ($p<0.002$) of total WBCs in knockout animals compared to their baseline levels. There was a significantly greater number of neutrophils ($p<0.02$) and lymphocytes ($p<0.05$) present in the blood of the knockout animals at this time point.

Figure 4 demonstrates the data of peripheral leukocytes in the wild-type animals at 24 hours post-burn compared to an uninjured state / baseline reading. While total white blood cell counts, as well as neutrophil and lymphocyte counts remained unchanged, monocytes showed a significant increase compared to uninjured conditions ($p<0.04$).

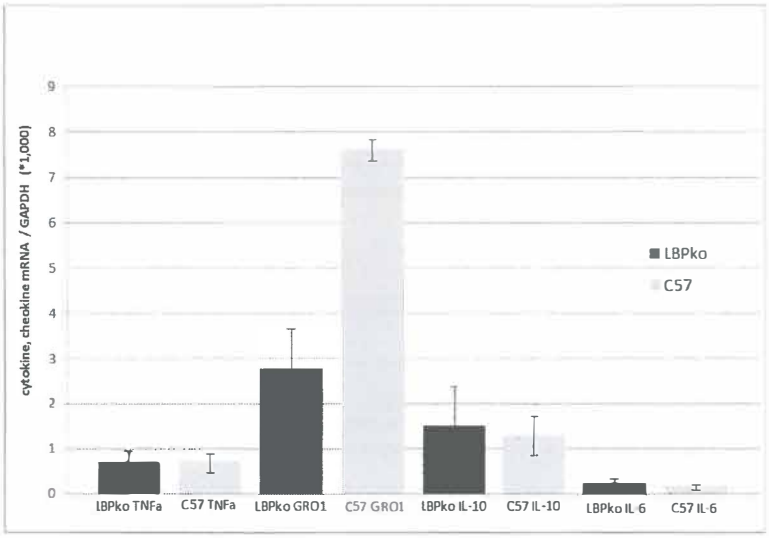

Figure 1: 24 hrs postburn comparison of intradermal gene expression levels of different cyto-/chemokines in LBPko versus wild type mice.

Skins were harvested 24h after burn infliction, and mRNA levels in these samples were related to GAPDH expression by real time RT-PCR. TNFα, GRO-1 and the interleukins IL-10 and IL-6 showed no significant difference between LBP ko and wt (C57) controls (bars represent 6 animals/group, +/– 1 SE).

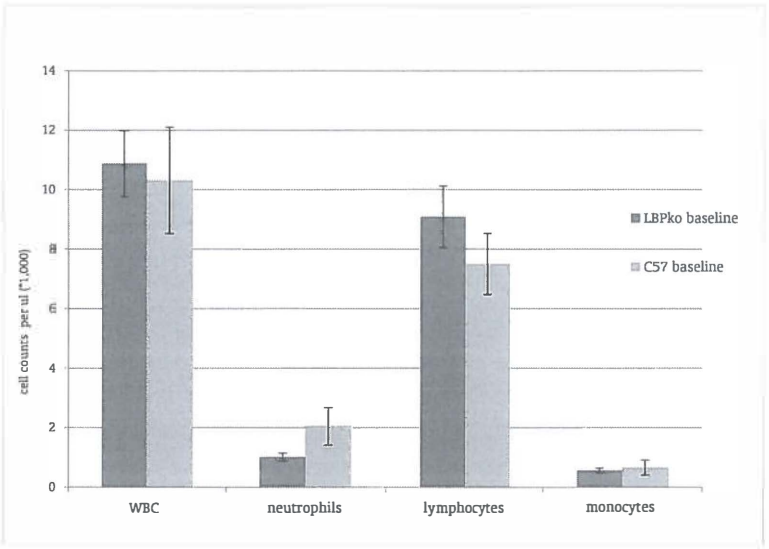

Figure 2: Results of total white blood cell and differential leukocyte number analysis of uninjured LBP knockout animals versus uninjured wild types.

Prior to wound infliction, the total WBC count and subset distribution of neutrophils, lymphocytes and monocytes were determined, comparing LBPko versus wt C57BL/6 controls (C57) in an uninjured state. No significance was found (each bar represent 6 animals, +/– 1 SD).

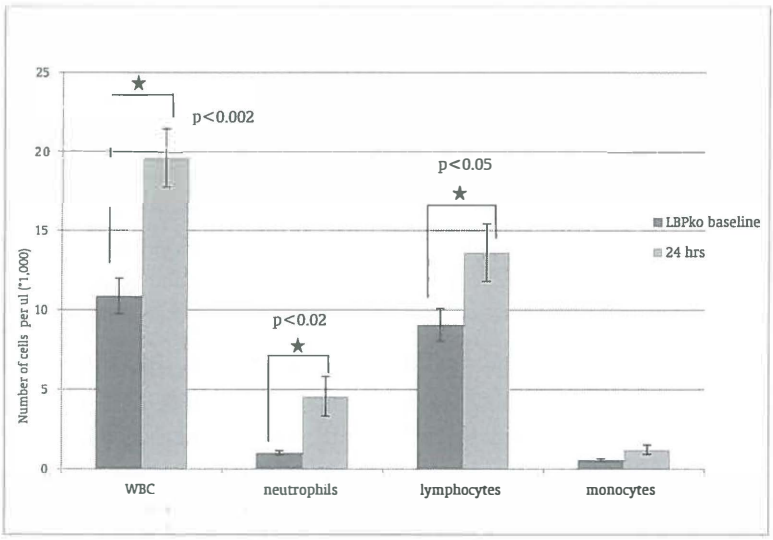

Figure 3: White blood cell and leukocyte subset counts at 24h after burn infliction in LBPko mice.
Comparison of white blood cell (WBC) and leukocyte subset counts between LBPko animals at uninjured state (baseline) and at 24 hours following burn. WBC almost doubled ($p<0.002$), the number of neutrophils ($p<0.02$) and lymphocytes ($p<0.05$) were significantly increased. Asterisk sign indicates significance (n=6 animals per group, +/– 1 SD).

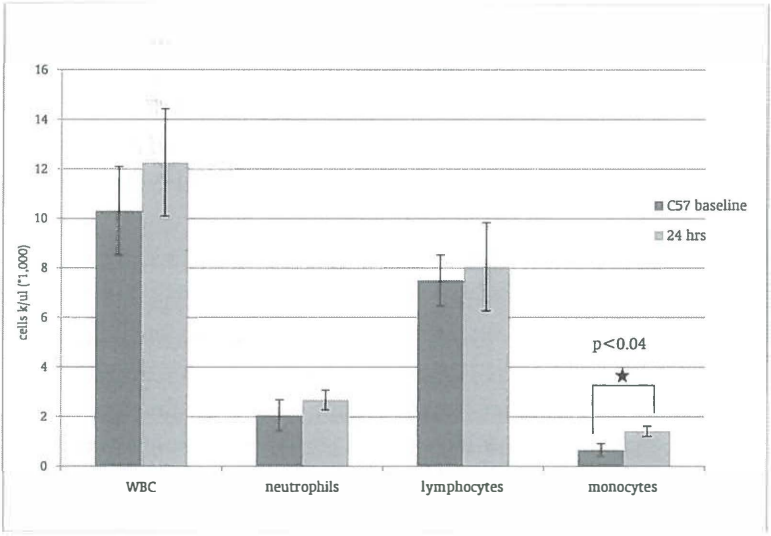

Figure 4: White blood cell and leukocyte subset counts at 24h after burn infliction in C57bl/6 wild type mice.
Comparison of total white blood count and leukocyte subsets of wild-type animals at baseline (C57 baseline, i.e., uninjured) and of mice 24 hours following partial thickness burn (24 hrs). Monocyte numbers had increased significantly ($p<0.04$). Asterisk sign indicates significance (n=6 animals per bar, +/- 1 SD).

Discussion

Our research is aimed at investigating the effects of LBP in deep partial thickness burns using an LBP-knockout mouse colony compared to corresponding wild types. We therefore subjected LBP-knockout and wt mice to burn trauma followed by Affimetrix® gene-chip analysis 6 hours post-burn in order to investigate possible changes in intradermal gene expression patterns. The highest level of gene expression was found for the CXC-chemokine GRO-1, with a more than 52-fold up-regulation in knockouts compared to controls. Out of the 36,000 genes analyzed, we found 12,500 to be altered with 1,802 of which showing a 2-fold difference, and 49 linked to inflammation. Quantitative RT-PCR data of pro- and anti-inflammatory cytokines generated at 24 hours after the burn revealed no difference between the two groups, whereas the GRO-1 gene expression level was higher in wt mice at this later point in time. When we compared total leukocyte and differential white blood cell counts of mice at an uninjured state, no differences were found between both groups. However, peripheral leukocytes almost doubled 24 hours following burn in knockouts, when compared to the uninjured state, with neutrophils and lymphocytes increased. In contrast, wild type animals reacted with monocytosis 24 hours after the burn and showed no alteration in the total number of leukocytes.

Gene-chip analysis using hybridization arrays has advantages and disadvantages. Some regard this technology as expensive 'non-hypothesis-driven' (21, 22), others as a very useful tool of functional genomics (23, 24). We deliberately chose to analyze at 6 hours post-trauma since Endlich and co-workers (25) described this particular time-point appropriate with special regard to inflammatory cytokines. The analysis of 36,000 genes of our experiment resulted in only 1,802 (5%) showing an at least two fold difference in expression level, when compared to the other animal group, out of which we associated 49 with inflammation. We expected inflammatory cytokines to be most likely affected, since the trigger for possibly altered gene-expression profiles in both groups, was thermal injury. By means of a gene chip analysis we aimed at shedding more light onto possible differences in genetic expression profiles of organisms able or unable to produce LBP, after having sustained a partial thickness burn of considerable size. GRO-1, a murine CXC chemokine analogue of human IL-8, was by far the most extensively up-regulated gene in partial thickness burn wounds of LBP-knockout mice. Interestingly, one of the GRO-1 binding receptors, CXCR-2, which plays a role in cutaneous wound healing (26), was 2-fold down-regulated in knockout mice. In order to quantify the data gained by the gene-chip, we conducted quantitative RT-PCR on inflammatory cytokines at 24 hours post-burn. Cytokines analyzed and known to play a role in cutaneous inflammation (27) were TNFα, IL-10 and IL-6 as well as GRO-1, with IL-6 also being altered as seen

in our Affimetrix® data. At this later point of time, the differences were not found to be significant as opposed to the 6 hour time-point.

Chemokines such as the CXC-chemokine GRO-1 found in our experimental model, are produced by leukocytes and tissue cells either constitutively or after stimulation (28) and are responsible for leukocyte chemoattraction (29). Due to overlapping function and only relative specificity of receptor affinity, a complex picture of CXCRs (receptors) binding CXCLs (ligands) *in vivo* prevails, making multiple options of neutrophil recruitment possible (30-33). According to Dunican and coworkers, CXC chemokines IL-8 and GRO- contribute to inflammatory reactions and suppress PMN apoptosis (34). By blocking the CXC receptor, neutrophil recruitment can be inhibited as also described by Dunican (35). When related to our data, we have found evidence that in LBP-knockout mice the GRO-1-receptor CXCR2 was down regulated in contrast to the up regulated GRO-1, which adds to Dunican´s discussion about relative specificity of receptor affinity. In a wide variety of cells the production of IL-8 or GRO can be induced by inflammatory cytokines at the transcriptional level (36, 37), therefore there is clear evidence that these chemokines are linked to inflammation and orchestrate leukocyte responses. As both, GRO-1 and the binding receptor CXCR2 were altered in LBP-knockout mice used in our model, we continued to investigate on neutrophils.

At first, we looked at neutrophil concentration in whole blood at an uninjured state, and compared the findings to 24 hours after partial thickness burn in both animal groups. It is well known that tissue injury results in an acute inflammatory reaction, mediated by cytokines and chemokines. Neutrophils, as key mediators of inflammation, usually constitute nearly 50% of all cells at a wound site, and appear at day 1 after tissue injury (38). In our experiment, we found no significant difference in the total number of leukocytes and specifically neutrophils, in either mouse group at an uninjured state. Twenty-four hours after partial thickness burn, the picture had changed. Knockout animals had almost doubled their numbers of systemically circulating leukocytes compared to their uninjured state. Significantly more neutrophils and lymphocytes were found in the knockouts as compared to the baseline reading, whereas wild types responded to burn infliction by increased monocytes under the same condition. Neutrophils represent an important subset of the leukocyte population, are short-lived, polymorphonuclear cells that play a key role in the early stages of the inflammatory response to infection by their ability to migrate, phagocytose, produce reactive oxygen species and also kill extracellular microbial pathogens. They are also key components of cutaneous wound healing (26), making them especially interesting for an experimental setting such as our burn wound model. During infection however, neutrophils leave the blood under the influence of chemotactic factors like microbial products (fMLP), certain complement components such as C5a, and chemokines such as IL-8, or as in this case, the mouse equivalent GRO-1. With reference to burn wounds or photo-ageing (39), some investigators found that the clear-

ance mechanisms of neutrophils by apoptosis are impaired in burned skin, caused by an extension of their life, which are due to local elevation of cytokine levels and the hypoxic conditions (40-42). In our experimental setting, the infliction of a burn had pronounced consequences on peripheral leukocyte and leukocyte subset numbers, and changed intradermal gene expression patterns of mice lacking the ability to produce a protein found important in Gram-negative infection.

Conclusion

In a standardized partial thickness burn wound model, profound alterations in intradermal gene expression levels were found between LBPko and wild type mice. Intradermally, the neutrophil chemoattractant GRO-1 was found to be the highest induced gene in LBPko mice analyzed by a gene-chip, early after burn injury. 24 hours after burn, no significant differences in intradermal pro- and anti-inflammatory cytokine gene expression levels were found between LBPko and wt mice using RT-PCR. When mice lack the ability to produce LBP, sustain major burn trauma even without infection, at 24 hours post-burn they react with neutrocytosis and lymphocytosis, in contrast to wild type mice demonstrating monocytosis. This observation implies that substantially different cellular immune strategies prevail in the presence respectively absence of LBP. Whether this neutrocytosis is GRO-1 dependent, and whether it leads to enhanced neutrophil migration into the affected skin, needs to be established, including the kinetics of the processes involved.

References

1. Bloemsma GC, Dokter J, Boxma H, et al. Mortality and causes of death in a burn centre. *Burns* 2008;34:1103-1107.
2. American Burn Association Resources Factsheet. Available at: http://www.ameriburn.org/resources_factsheet.php.
3. National Burn Registry Annual Report 2010. Available at: http://www.ameriburn.org/2010NBRAnnualReport.pdf.
4. Muller MJ, Herndon DN. The challenge of burns. *Lancet* 1994;343:216-220.
5. D'Avignon LC, Hogan BK, Murray CK, et al. Contribution of bacterial and viral infections to attributable mortality in patients with severe burns: an autopsy series. *Burns* 2010;36:773-779.
6. Ravat F, Le-Floch R, Vinsonneau C, et al. Antibiotics and the burn patient. *Burns* 2 011;37:16-26.
7. Rezaei E, Safari H, Naderinasab M, et al. Common pathogens in burn wound and changes in their drug sensitivity. *Burns* 2011;37:805-807.
8. Klein RD, Su GL, Aminlari A, et al. Skin lipopolysaccharide-binding protein and IL-1beta production after thermal injury. *J Burn Care Rehabil* 2000;21:345-352.
9. Tobias PS, Soldau K, Ulevitch RJ. Isolation of a lipopolysaccharide-binding acute phase reactant from rabbit serum. *J Exp Med* 1986;164:777-793.
10. Tobias PS, Mathison J, Mintz D, et al. Participation of lipopolysaccharide-binding protein in lipopolysaccharide-dependent macrophage activation. *Am J Respir Cell Mol Biol* 1992;7:239-245.
11. Su GL, Freeswick PD, Geller DA, et al. Molecular cloning, characterization, and tissue distribution of rat lipopolysaccharide binding protein. Evidence for extrahepatic expression. *J Immunol* 1994;153:743-752.
12. Vianello F, Righi E, Poznansky MC. Methods for quantitation of leukocyte chemotaxis and fugetaxis. *Methods Mol Biol* 2010;616:115-124.
13. Pease JE, Williams TJ. Chemokines and their receptors in allergic disease. *J Allergy Clin Immunol* 2006;118:305-318; quiz 319-320.
14. Ansel KM, Ngo VN, Hyman PL, et al. A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature* 2000;406:309-314.
15. Wurfel MM, Monks BG, Ingalls RR, et al. Targeted deletion of the lipopolysaccharide (LPS)-binding protein gene leads to profound suppression of LPS responses ex vivo, whereas in vivo responses remain intact. *J Exp Med* 1997;186:2051-2056.
16. Gilpin DA. Calculation of a new Meeh constant and experimental determination of burn size. *Burns* 1996;22:607-611.
17. Giordano TJ, Thomas DG, Kuick R, et al. Distinct transcriptional profiles of adrenocortical tumors uncovered by DNA microarray analysis. *Am J Pathol* 2003;162:521-531.
18. Giordano TJ, Shedden KA, Schwartz DR, et al. Organ-specific molecular classification of primary lung, colon, and ovarian adenocarcinomas using gene expression profiles. *Am J Pathol* 2001;159:1231-1238.
19. Lipshutz RJ, Fodor SP, Gingeras TR, et al. High density synthetic oligonucleotide arrays. *Nat Genet* 1999;21:20-24.

20. Su GL, Hoesel LM, Bayliss J, et al. Lipopolysaccharide binding protein inhibitory peptide protects against acetaminophen-induced hepatotoxicity. *Am J Physiol Gastrointest Liver Physiol* 2010;299:G1319-1325.
21. Brenner S. Sillycon valley fever. *Curr Biol* 1999;9:R671.
22. Mir KU. The hypothesis is there is no hypothesis. The Microarray Meeting, Scottsdale, Arizona, USA, 22-25 September 1999. *Trends Genet* 2000;16:63-64.
23. Sellheyer K, Belbin TJ. DNA microarrays: from structural genomics to functional genomics. The applications of gene chips in dermatology and dermatopathology. *J Am Acad Dermatol* 2004;51:681-692; quiz 693-686.
24. Brown PO, Botstein D. Exploring the new world of the genome with DNA microarrays. *Nat Genet* 1999;21:33-37.
25. Endlich B, Armstrong D, Brodsky J, et al. Distinct temporal patterns of macrophage-inflammatory protein-2 and KC chemokine gene expression in surgical injury. *J Immunol* 2002;168:3586-3594.
26. Zaja-Milatovic S, Richmond A. CXC chemokines and their receptors: a case for a significant biological role in cutaneous wound healing. *Histol Histopathol* 2008;23:1399-1407.
27. Zhu M, Oishi K, Lee SC, et al. Studies using leukemia inhibitory factor (LIF) knockout mice and a LIF adenoviral vector demonstrate a key anti-inflammatory role for this cytokine in cutaneous inflammation. *J Immunol* 2001;166:2049-2054.
28. Baggiolini M. Chemokines in pathology and medicine. *J Intern Med* 2001;250:91-104.
29. Kobayashi Y.. The role of chemokines in neutrophil biology. *Front Biosci* 2008;13:2400-2407.
30. Tsai WC, Strieter RM, Wilkowski JM, et al. Lung-specific transgenic expression of KC enhances resistance to Klebsiella pneumoniae in mice. *J Immunol* 1998;161:2435-2440.
31. Tessier PA, Naccache PH, Clark-Lewis I, et al. Chemokine networks in vivo: involvement of C-X-C and C-C chemokines in neutrophil extravasation in vivo in response to TNF-alpha. *J Immunol* 1997;159:3595-3602.
32. Nakagawa H, Ando Y, Takano K, et al. Differential production of chemokines and heir role in neutrophil infiltration in rat allergic inflammation. *Int Arch Allergy Immunol* 1998;115:137-143.
33. Nagarkar DR, Wang Q, Shim J, et al. CXCR2 is required for neutrophilic airway inflammation and hyperresponsiveness in a mouse model of human rhinovirus infection. *J Immunol* 2009;183:6698-6707.
34. Dunican A, Grutkoski P, Leuenroth S, et al. Neutrophils regulate their own apoptosis via preservation of CXC receptors. *J Surg Res* 2000;90:32-38.
35. McColl SR, Clark-Lewis I. Inhibition of murine neutrophil recruitment in vivo by CXC chemokine receptor antagonists. *J Immunol* 1999;163:2829-2835.
36. Matsushima K, Baldwin ET, Mukaida N. Interleukin-8 and MCAF: novel leukocyte recruitment and activating cytokines. *Chem Immunol* 1992;51:236-265.
37. Haskill S, Peace A, Morris J, et al. Identification of three related human GRO genes encoding cytokine functions. *Proc Natl Acad Sci U S A* 1990;87:7732-7736.
38. Engelhardt E, Toksoy A, Goebeler M, et al. Chemokines IL-8, GROalpha, MCP-1, IP-10, and Mig are sequentially and differentially expressed during phase-specific infiltration of leukocyte subsets in human wound healing. *Am J Pathol* 1998;153:1849-1860.

39. Rijken F, Bruijnzeel-Koomen CA. Photoaged skin: the role of neutrophils, preventive measures, and potential pharmacological targets. *Clin Pharmacol Ther* 2011;89:120-124.
40. Chitnis D, Dickerson C, Munster AM, et al. Inhibition of apoptosis in polymorphonuclear neutrophils from burn patients. *J Leukoc Biol* 1996;59:835-839.
41. Hannah S, Mecklenburgh K, Rahman I, et al. Hypoxia prolongs neutrophil survival in vitro. *FEBS Lett* 1995;372:233-237.
42. Steevels TA, Meyaard L. Immune inhibitory receptors: essential regulators of phagocyte function. *Eur J Immunol* 2011;41:575-587.

Chapter 4

Lipopolysaccharide-binding protein (LBP) deficiency in a mouse burn-wound model results in systemically and topically altered immune status

4

Abstract

Introduction:
Previously, a burn wound-infection model was created to investigate innate immunity and host defense against Gram-negative infection. Application of this model to lipopolysaccharide-binding protein (LBP) deficient mice allowed studying the role of this protein in immunological reactions of burn wound infections. The purpose of the current study was to investigate topical as well as systemic immunological changes in cellular behavior and mRNA expression levels in reaction to the burn, over a period of 96 hours. In addition, we studied the natural bacterial colonization difference between LBP knockout and control mice.

Methods:
LBP-knockout mice and corresponding wild type controls were subjected to a deep partial thickness burn. At 6, 24, 48 and 96 hours following trauma, intradermal mRNA profiling was conducted, focusing on the chemokine GRO-1, as well as pro- and anti-inflammatory cytokines. Peripheral blood analysis was performed for total white blood cells and leukocyte subsets. A myeloperoxidase (MPO) assay was conducted to measure neutrophil oxidative burst intradermally in both groups. The natural bacterial colonization in either mouse colony was studied 6 days following the burn wound after having left the burn uncovered for the whole period.

Results:
GRO-1, TNFα and IL-10 showed a biphasic expression profile in time, with no difference between both groups. In contrast, IL-6 exhibited a monophasic profile, with at the later time points significantly lower levels in LBPko mice. At 24 and 96 hours following trauma, knockout animals had significantly more leukocytes in their peripheral blood compartment compared to wild types, at 96h this number was 4-times as high. In the MPO assay higher readings were obtained in wild-type skin at 24 and 96 hours compared to the readings in LBP knockout mice. Without wound coverage, the natural colonization process resulted in less bacteria on the skin of knockout mice 6 days after the burn.

Conclusion:
With this study, we showed that LBP-deficiency led to profoundly changed peripheral leukocyte subset numbers, intradermal GRO-1 and IL-6 mRNA expression levels, and neutrophil activation status. Furthermore, knocking out LBP affected the immunological response not only to Gram-negative bacteria as previously shown, but also to Gram-positive, when the burns were subjected to a natural bacterial re-colonialisation over a period of 6 days following burn.

Introduction

During the past decades, the focus of interest in burn wound infection has shifted from a macroscopic to a microscopic level, from the treatment of burn wound related infections to understanding the underlying cellular interactions between the host and the pathogen. The view has narrowed to a (sub)cellular, molecular level, resulting from a greater understanding of protein-receptor interactions and intracellular pathways necessary to adequately respond to infections.

Burn wound infection determines the clinical outcome of wound healing, the incidence of complications, and the overall result (1-5). Interestingly, Gram-positive bacteremia exerts no identifiable effect on the predicted mortality of burn patients, whereas Gram-negative bacteremia significantly increases the death rate above that predicted on the basis of extent of burn and patient age (6). In a recent study, factors found to associate with burn mortality included age, total and full thickness burn-surface area, female gender and early mechanical ventilation (7). Interestingly, infection-related death is independent of the percentage of total body surface area burn, the percentage of full thickness burn and inhalation injury (8). Gram-negative burn wound infections are associated with high morbidity and mortality (8-10), with *Pseudomonas aeruginosa* being the most feared bacterium (11). According to Azzopardi and coworkers (2), these Gram-negative infections are most challenging due to their ubiquity in the natural environment, their high prevalence of multidrug resistance and rapid acquisition of resistance causing high death rates. Various authors describe high mortality directly or indirectly resulting from post-burn infections (6, 8, 11, 12).

Lipopolysaccharide-binding protein (LBP) represents a central participant in host immune response to Gram-negative infection. LBP binds to the lipid A portion of bacterial lipopolysaccharide (LPS, endotoxin), the principal component of the outer membrane of Gram-negative bacteria (13). This protein is produced not only in the liver, but also in various extrahepatic sites, such as wounds (14). LBP enhances binding of LPS to membrane-bound CD14 (15), a GPI-anchored protein without transmembrane components. The Toll-like receptor family transmits the LBP-mediated LPS-CD14 signal to intracellular signaling pathways leading to gene transcription activity (16-18). Previously, we have demonstrated an inverse relationship between LBP expression in burns and the number of dermal bacterial colonies (19). Next to LBP as an acute phase protein, interleukin production is triggered by scald injuries as well (20, 21). By investigating selected pro-inflammatory cytokines, like interleukine-1 (IL-1), IL-6 and tumor-necrosis factor alpha (TNFα), as well as anti-inflammatory cytokines with opposing activity like IL-10, an adequate description of the cytokine-network initiated by burn wound infections is possible (22, 23).

We had homozygous LBP-knockout (LBPko) mice available allowing to obtain further insight into LPS-LBP interaction which initiates the immune response within Gram-negative infected burn wounds. Earlier, we have shown the importance of LBP using these knockout mice in a standardized partial thickness burn model when infected with *Pseudomonas aeruginosa*. We demonstrated that local administration of an adenovirus construct encoding for LBP enhanced immunity at the site of infection. The burn wound itself is of utmost importance in this context. At the site of burn, intradermal cytokine as well as chemokine gene expression (24) is increased, triggering further inflammatory cytokine production and cellular chemo-attraction. Consequently, cells of the immune system respond and migrate towards the site of injury (25, 26), participate in a topical and subsequently systemic inflammatory reaction, with leukocytes orchestrating the immune response. Previously, in a series of experiments, we found no differences in peripheral blood leukocyte numbers between wild type (wt) and LBPko mice at an uninjured state. However, after having sustained a partial thickness burn, the picture had changed (*chapter 3 of this thesis*). At 24 hours following the burn injury, knockout mice had almost doubled their peripheral leukocytes and neutrophils, while also lymphocyte numbers had increased, whereas wild types mice showed increased monocyte numbers only. As another consequence of the burn, LBPko mice reacted to thermal injury with the expression of the chemokine growth-related oncogene-1 (GRO-1) known to attract neutrophils, earlier and to a higher degree than wt mice.

With the experiments described here, we focused on monitoring pro- and anti-inflammatory cytokine mRNA levels using quantitative reverse-transcription polymerase-chain reaction (qRT-PCR), at up to 96 hours following partial thickness burn. We hypothesized that neutrophils would find their way in response to intradermal GRO-1 expression, discovered in previous experiments to be extensively up regulated at 6h and 24h postburn, in both LBPko and wt mice. Therefore, we monitored systemic leukocytes including their subsets at up to 96 hours as well as studied neutrophil accumulation intradermally, in both wt and LBPko groups. Furthermore, we hypothesized that without inflicting a defined Gram-negative infection, intradermal neutrophil accumulation would be capable of influencing the natural course of a bacterial re-colonialisation of our standardized burn wound. Therefore we subjected LBP-knockout mice and wild-types to the partial thickness burn, and had their wounds uncovered for 6 days followed by quantitative bacterial counts.

Materials, Methods

Animals

LBP knockout mice were a gift from Douglas Golenbock (Boston University School of Medicine) (27). We had the animals backcrossed at least 12 times into their C57BL/6 background-strain prior to use. All experiments were performed according to the regulations of the National Institute of Health guidelines for the use of laboratory animals and approval was obtained from the University of Michigan Animal Care and Use Committee. Mice were kept in specific pathogen-free environment under a 12-hour light cycle, fed with rodent chow and water ad libidum in micro-isolator containments. For all experiments, we used 12-14 weeks old female, weight- and/or age-matched mice. C57BL/6 wt animals were purchased from Harlan (Harlan, Indianapolis, IN), allowing at least a 5 day period of acclimatization prior to experiments.

Burn model, without infection

Twenty-four female wt mice from Harlan (Harlan, Indianapolis, IN) and 24 in-house bred female LBPko mice were age/weight–matched. Out of these, 4 random groups of knockouts and wild-types were formed. All animals were clipped at their dorsum under general anesthesia (Ketaset® (Ketamine HCl, 100mg/ml, Fort Dodge, Fort Dodge, IO), AnaSed® (Xylazine 20mg/ml, Lloyd Laboratories, Shenandoah, IO)) applied intraperitoneally (ip). After depilation (Nair®, Carter Products, New York, NY), the animals were placed in a mold exposing 25% of their total body surface area at their dorsum in a hot water bath by partially submerging (60° Celsius for 10s), creating a deep partial thickness burn wound. The desired burn wound size was determined by the Meeh formula (28) before infliction. Immediately postburn the skin was dried and animals resuscitated with ip saline, and buprenorphine (Buprenex®, Reckitt & Colman, Richmond, VA) was injected subcutaneously every 6-8 hours for 2 days well away from the burn site. Next, six animals of each group (LBPko and wt) were euthanized after 6, 24, 48 and 96 hours by an overdose of ip Pentobarbital (Sodium Pentobarbital 50mg/ml, Abbott Laboratories, North Chicago, IL) to be subjected to dermal tissue harvest (for RNA extraction for qRT-PCR analysis and MPO analysis). Peripheral blood samples were also taken at the 24, 48 and 96 hour time point to be analyzed for peripheral leukocytes and leukocyte subsets (Hemavet®).

Burn model plus natural bacterial colonization

Another twelve animals, six wt versus 6 LBPko mice, were burned and left with uncovered wounds without applying a defined infection and therefore subjected to a natural

course of bacterial re-colonization. Resuscitation and pain management was the same as described above. Quantitative bacterial counts were conducted on day 6 postburn. Burned skin was harvested under sterile conditions, weighed and homogenized in saline on ice. Homogenates were plated in serial dilutions in triplicates (10^2, 10^4, 10^6) on 5% sheep-blood agar plates. The following day, bacterial colonies were counted in a blinded fashion and standardized per g/skin, presented as calculated mean of plates (+/- 1 standard error, SE), out of triplicates. Bacterial typing was done by morphology.

Gene expression analysis by real time RT-PCR

All skin samples were homogenized without being thawed in the presence of TRIzol reagent (Life Technologies Inc., Gaithersburg, MD) and total cellular RNA was extracted according to the manufacturer's guidelines. The samples were then subjected to the RNeasy® preparation protocol for spin columns (Qiagen, Valencia, CA). Reverse transcription was carried out using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time PCR was carried out using the IQ SYBR Green Supermix and the SmartCycler® (Cepheid, Sunnyvale, CA).

The GRO-1, TNFa, IL-10 and IL-6 quantitative RT-PCR (SmartCycler®, Cepheid, Sunnyvale, CA) included a denaturation step for 120 sec at 95°C and 3 temperature-cycles repeated 45 times (94.0°C for 15s, 64.0°C for 15s, 72.0°C for 40s) followed by a melting curve from 60.0°C to 95.0°C at 0.2°C/second for each sample. IL-6 primers were received from R+D Systems Inc. (Minneapolis, MN), mouse IL-10 sense primer was: CCAGTTTTACCTGGTAGAAGTGATG, IL-10 antisense primer: TGTCTAGG CCTGGAGTCCAGCAGACTC, mouse TNF sense primer CTGTAGCCCACGTCGTAGC, and antisense primer TTGAGATCCATGCCGTTG. GRO-1 primers were designed as described in *chapter 3* of this thesis: forward /5' sequence: CAA TGA GCT GCG CTG TCA GTG, sense, reverse / 3' sequence: AAG CCT CGC GAC CAT TCT TGA, antisense. PCR products were also examined using a 1% agarose ethidium-bromide gel to confirm the correct size of the PCR product for each primer set. Our target gene threshold cycle (Ct) was compared to a housekeeping gene of the same sample (glyceraldehyde-3-phosphate dehydrogenase, GAPDH), results are reported as ratios of target gene Ct / GAPDH Ct (29).

Myeloperoxidase (MPO) assay of skin samples

Neutrophil infiltration and presence in the skin was quantitated by measuring MPO activity according to a modification of the Goldblum-protocol (30, 31). Skin samples were homogenized for 1 minute on ice in homogenization buffer (HTAB, hexadecyl-trimethylammonium bromide), 0.5M EDTA, mono- and dibasic potassium phosphate and H_2O).

Next, the samples were continuously sonicated three times for 10 seconds, 1 ml was transferred and spun at 15,000 rpm for 15 min. in 4° C. Assay buffer (containing mono- and dibasic potassium phosphate, 0.3% H_2O_2 and ODH (*o*-dianisidine HCL)) was prepared shortly before the assay. An ELISA plate reader was set to read the change in absorbance at 465 nm every 10s for 90s per well. Next, 20 µl of sample were pipetted into a 96 ELISA plate in triplicates per sample, 200 µl assay buffer per well were added and reading was started immediately thereafter. MPO units were calculated as the change in absorbance over a reading-period of 90 s (delta OD (optical density)) per well and mean of triplicate wells per sample were analyzed and calculated. Peroxidase activity was thus determined by measuring the rate of conversion of *o*-dianisidine to its colored product in the presence of H_2O_2.

Hemavet® blood analysis

Whole blood of LBPko and C57BL/6 wt controls was analyzed for peripheral white blood cell counts, including neutrophils, monocytes and lymphocytes at sacrifice. EDTA anti-coagulated blood samples were subjected to analysis in Hemavet® Mascot Multispecies Hematology System Counter 1500R (CDC Technologies, Inc., Oxford, CT). Samples were counted within five minutes after blood was drawn.

Statistical analysis

Statistical analysis was performed using StatView software (SAS Institute, Cary, NC). Data shown express mean value ± 1 SEM (standard error), comparison was performed using two-tailed Student's *t*-test, statistical significances was considered as $p<0.05$.

Results

In intradermal gene-chip analysis and qRT-PCR done earlier, we found altered gene expression levels of the chemokine GRO-1 as a response to burn trauma in both LBPko and wt mice. Here, intradermal mRNA levels of GRO-1 were obtained at 6, 24, 48 and 96 hours following partial thickness burn infliction (*Figure 1*). Using real time RT-PCR, GRO-1 expression levels were shown to follow a biphasic course, with the highest expression at 24 hours in both animal groups. At 6 hours, LBPko animals had a higher expression ($p<0.001$) than the wt, whereas at 24 hours wt animals showed higher GRO-1 expression ($p<0.01$). No difference was observed at 48 and 96 hours post-trauma. As seen in *Figure 2*, the inflammatory cytokine TNFa, as GRO-1, also showed a biphasic pattern, with no significance between the groups. When analyzing the gene-expression levels of the anti-inflammatory cytokine IL-10, the same biphasic expression and kinetics thereof was seen as observed for TNFα and GRO-1 (*Figure 3*). Also for IL-10, no significance was found when comparing the two groups. As demonstrated in *Figure 4*, IL-6 did not follow the biphasic patterns of the other inflammatory cytokines, neither in LBPko mice nor in wt controls. IL-6 levels of LBPko mice dropped approximately 10-fold below those of C57BL/6 mice at 48 and 96 hours after burn.

Besides cytokine profiles, we also studied leukocyte changes at 24, 48 and 96 hours after the burn. *Figure 5* shows the total peripheral white blood cell (WBC) counts of both wt and LBPko mice. At 96 hours following burn, the total number of WBC in the peripheral blood of LBPko animals was significantly, almost 4-times higher than in wild-types. As demonstrated in *Figure 6*, no difference was found in peripheral neutrophil numbers. Lymphocyte numbers (*Figure 7*) were found to be higher in LBPko animals at 24 and 96 hours. At 96 hours after burn, there were 4-times as many lymphocytes in knockout mice as there were in wt animals. As for monocytes at the 48-hour time point, LBPko animals had lower numbers, as shown in *Figure 8*.

In order to study neutrophil numbers and/or activation intradermally, we analyzed myeloperoxidase in skin extracts of both groups (*Figure 9*). This assay quantitates the oxidative burst of neutrophils. At 24 hours post burn, statistically significantly higher MPO readings were detected in the skin of wild type mice, suggesting the presence of more neutrophils or neutrophils with higher oxidative bursts intradermally in this group.

When the animal skin was left uncovered and therefore unprotected against contamination, there was less bacterial growth ($p=0.03$) on the skin of LBPko mice at 6 days post-burn compared to the growth on the skin of wt (*Figure 10*). The six day period revealed a mixture of Gram-positive bacterial infection namely of *Staphylococci* and *Streptococci.*

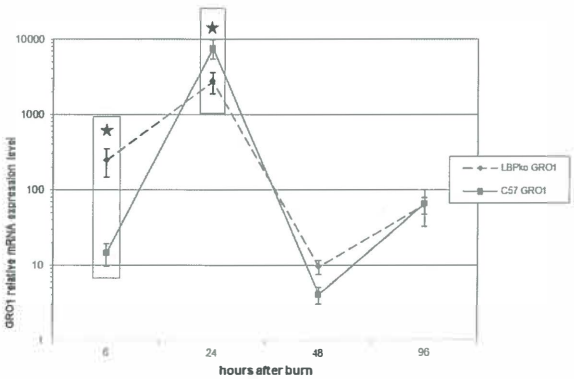

Figure 1: Intradermal GRO-1 mRNA expression levels in burned skin of LBPko mice compared to wt mice in time after burn trauma.

Animals were burned and GRO-1 mRNA samples of LBPko versus wt mice were taken at several time points, and mRNA levels were assessed by real time RT-PCR as described in Materials and Methods. At 6 and 24 hours after the burn, GRO-1 mRNA expression levels were statistically different. (n=6 animals / group). Asterisks indicate significance between the groups (6 hours $p<0.001$, 24 hours $p<0.01$, data show +/– 1SE).

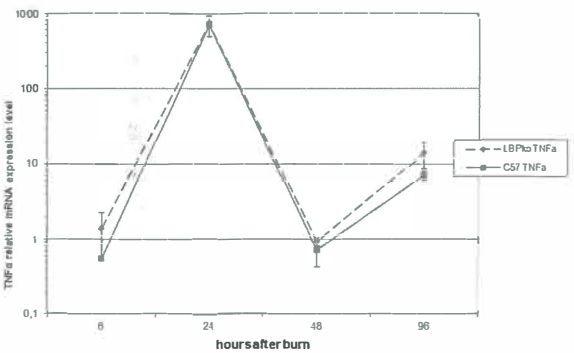

Figure 2: Intradermal TNFa mRNA expression levels in burned skin of LBPko and wt mice at several time points up to 96 hours after burn infliction.

At different time points after inflicting the standardized burn, TNFa mRNA levels were analyzed by real time RT-PCR as described in Materials and Methods. At 24 hours postburn, the maximum mRNA expression is seen in both groups. In time, wt (C57) and LBPko animals show comparable mRNA expression profiles in a biphasic course (n=6 animals per data point, data are presented with +/– 1SE).

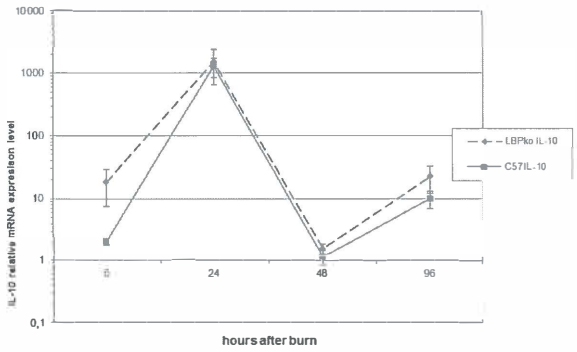

Figure 3: Intradermal IL-10 mRNA expression levels in burned skin of LBPko versus wt in time after burn trauma.

As described in Material and Methods, the IL-10 mRNA levels were assessed by qRT-PCR at different time points following standardized burn, comparing knockout and wt mice. The anti-inflammatory cytokine IL-10 revealed a peak at 24 hours and showed no difference in expression profiles and kinetics between the groups (n=6 animals per data point, +/– 1 SE).

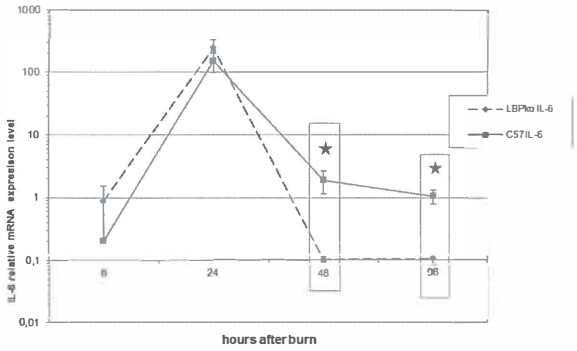

Figure 4: Intradermal IL-6 mRNA levels comparing knockout mice with wt in time after burn.

At different time points after inflicting the standardized burn, IL-6 mRNA levels were assessed by real time RT-PCR as described in Materials and Methods. The IL-6 mRNA levels remained higher at 48 and 96 hours in wt mice following burn compared to their levels in LBPko mice. Asterisks indicate significance ($p<0.05$ at 48 and 96 hours, n=6 animals per data point, +/– 1 SE).

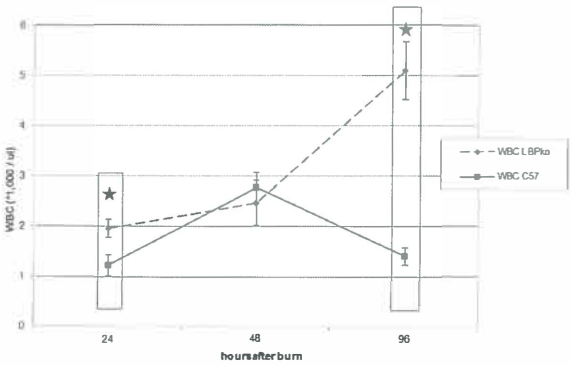

Figure 5: Analysis of total white blood cell counts in LBPko versus wt mice in time after burn infliction.

At different time points after inflicting the standardized burn, total white blood cell count was performed as described in Materials and Methods. At 24 and 96 hours following the trauma, LBPko animals have significantly more leukocytes (WBC) in their peripheral blood than the wt, at 96 hours this numbers is 4-times as high. Asterisks sign indicate significance ($p<0.05$, n=6 animals per group, +/– 1 SE).

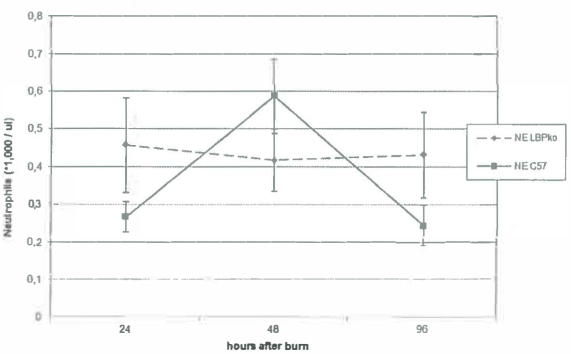

Figure 6: Peripheral blood neutrophil counts in LBP-knockout mice versus wt in time after burn infliction.

As described in Material and Methods, whole blood neutrophil numbers were assessed at different time points after inflicting the standardized burn. The neutrophil numbers (NE) were found to be not significantly changed between both groups at any given time point (n=6 per data point, +/– 1 SE).

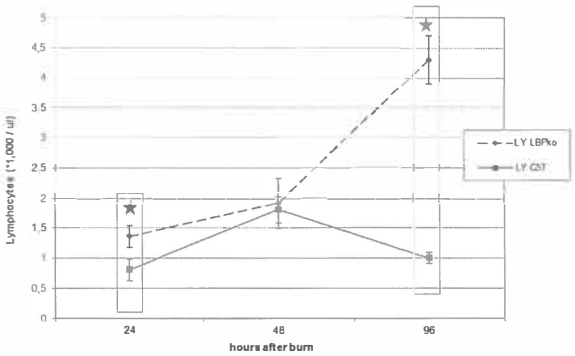

Figure 7: Peripheral blood lymphocyte cell counts in LBPko mice versus wt in time after burn infliction.

At different time points after inflicting the standardized burn, whole blood lymphocyte numbers were assessed as described in Materials and Methods. More peripheral lymphocytes (LY) were found in knockout mice at 24 and 96 hours following burn compared to wt controls. At 96 hours following burn, there were approximately 4-times as many lymphocytes in the blood of knockouts than in blood of controls. Asterisks indicate significance ($p<0.05$, n=6 animals per data point, +/– 1 SE).

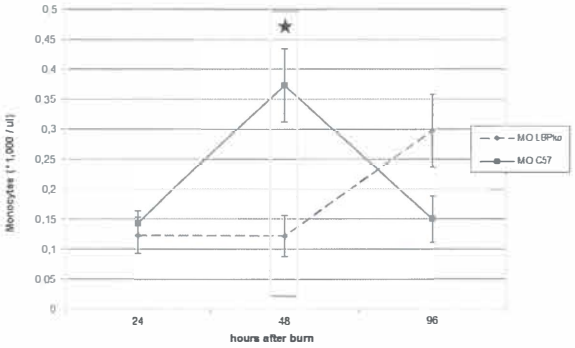

Figure 8: Peripheral blood monocyte counts of LBPko versus wt at up to 96 hours after burn.

At different time points after inflicting the standardized burn, whole blood monocyte numbers were assessed as described in Materials and Methods. At 48h after burn, there were more monocytes in the blood of wt mice compared to LBPko. Asterisk sign indicates significance ($p<0.05$, n=6 animals per data point, +/– 1 SE).

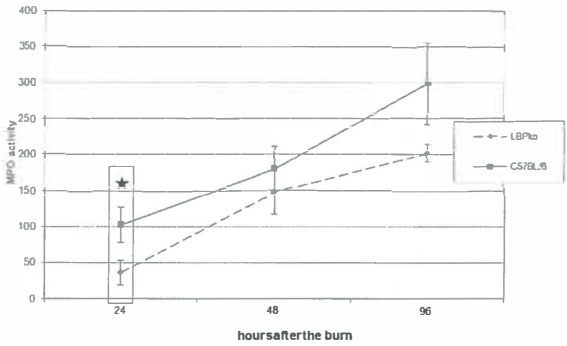

Figure 9: Myeloperoxidase assay analysis of skin samples of LBPko mice versus wt subjected to partial thickness burns at up to 96 hours after burn.

Skin samples of mice were subjected to an MPO assay as described in Materials and Methods. The MPO assay allows for detection of oxidative bursts of neutrophils. At 24 hours, MPO readings were significantly higher in C57BL/6 wt mice compared to LBPko. Asterisk sign indicates significance ($p<0.05$, n=6 animals per data point, +/– 1 SE).

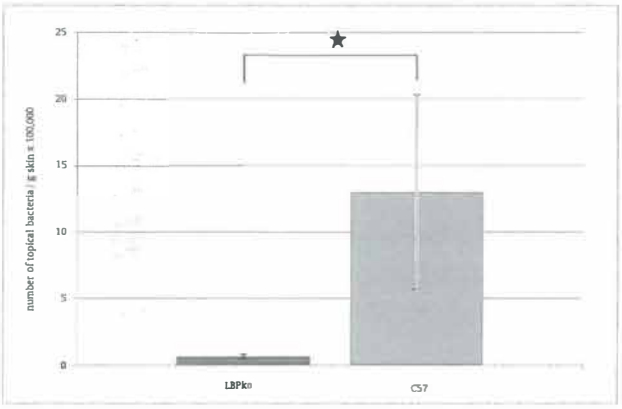

Figure 10: Comparison of bacterial growth on uncovered skin of LBP knockout mice versus wt at 6 days post burn.

In order to study the natural re-colonialisation of burned mouse skin, the animals were burned as described in Materials and Methods, leaving their wounds uninfected or covered by sterile dressings. The bacterial re-colonization revealed mostly Gram-positive bacteria and showed increased numbers on the skin of wt mice, i.c., 20.6-fold more bacteria than on knockout skin. Asterisk indicates significance ($p=0.03$ +/– 1 SE, n=5 animals per group)

Discussion

Earlier, we developed a partial thickness burn model in LBP-knockout and wt control mice to study Gram-negative infection. As a result of dermal gene chip analysis and qRT-PCR we had found altered intradermal gene expression profiles of the chemokine GRO-1 at 6 hours and 24 hours post-burn. With the experiments described here, we focused on monitoring pro- and anti-inflammatory cytokine mRNA levels up to 96 hours. GRO-1, as well as IL-10 and TNFα showed a biphasic pattern of mRNA expression, whereas IL-6 demonstrated a monophasic pattern. IL-6 revealed an almost 10-fold lower expression in LBPko mice at 48 and 96 hours, whereas TNFα and IL-10 did not show significant difference between the two groups of mice. GRO-1 is known for neutrophil chemoattraction, therefore systemic leukocyte numbers including their subsets at up to 96 hours were monitored, as well as neutrophil accumulation intradermally at the site of burn in both wild type and LBP knockout mice. 96 hours post burn, LBPko mice had almost doubled their total WBC numbers in peripheral blood, and demonstrated lymphocytosis, whereas knockout mice had less monocytes at 48 hours after burn. For neutrophil detection, an MPO assay was performed on burned mouse skin and showed lower readings in LBPko at 24 hours. Considering the role of chemokines and neutrophils in topical bacterial clearance in our burn model, we were interested in the natural course of bacterial re-colonialisation from surrounding skin. We thus left their burn wounds uncovered for 6 days, followed by quantitative bacterial counts, which showed especially Gram-positives on both wounds and less bacterial numbers on knockout skin.

In the current set of experiments, we found biphasic mRNA expression levels of TNF and IL-10, as well as of GRO-1. Not only GRO-1, but also the pro- and anti-inflammatory cytokines studied, demonstrated their maximum mRNA expression levels at 24 hours after the injury. With regard to the inflammatory process of wound repair / regeneration, it is accepted that cytokine expression induction occurs in a time dependent manner and is subsequently followed by cellular inflammatory reactions (32-34). According to Feezor and co-workers (35), cutaneous gene expression after a second degree burn follows a transient and time-dependent regulation, peaking at 3 and 14 days after the burn. When each cytokine, including GRO-1, was analyzed individually over the period of 96 hours, our results indicated a peak expression at 24 hours post-burn in contrast to Feezor´s results. IL-6 was the only cytokine showing a difference in expression kinetics between wt and knockouts, being higher expressed at 48 and 96 hours in wt than in LBP-knockout animals. Severe burns *per se* are thought to act in two inflammatory phases in a time dependent manner and therefore initiate an immune response. The first reaction of the organism is of a systemic inflammatory response syndrome (SIRS), followed by an anti-inflammatory phase (CARS) characterized by a profound immune-deficiency through

cellular deprivation (36), which can be seen as a biphasic response (37). The time-course of both, especially with regard to our knockout model, is unknown. In our current study we have noted a biphasic response in TNFα, IL-10, and GRO-1 at 24 and 96 hours after the burn, which fits well with the biphasic, but time wise unpredictable SIRS – CARS phenomenon (38).

When subjecting our burn model to commercially available gene-chip analysis at 6 hours post-burn, the chemokine GRO-1 showed a striking intradermal up-regulation in LBPko mice compared to wt under the same condition. Since GRO-1 is known to chemoattract neutrophils, we were interested in whether this increased GRO-1 expression early in the reaction to burn, persisted and was possibly associated with increased accumulation of neutrophils at the site of GRO-1 expression, leading to higher numbers of neutrophils in the dermis of knockout mice after being burned. Leukocytes follow chemokine mRNA expression with a certain delay. We have therefore chosen several different time points to study peripheral total white blood cell and leukocyte subset counts. The time-line we have chosen is in accordance with Lederer's group (39), who focused on leukocyte gene expression profiles in the peripheral blood pool at 2 hours, 1 day, 3 days and 7 days following a scald injury or sham. In their study, the authors concluded that the highest gene expression level of mouse leukocytes was seen at 1 day following trauma.

The chemokine GRO-1 is believed to be the murine analogue of human IL-8 (40, 41) known for neutrophil chemoattraction. Neutrophils play a central role in modulating and orchestrating the immune response triggered by invading pathogens, and were of interest to investigate in our model since GRO-1 expression kinetics was strikingly altered due to the absence of LBP. An MPO assay makes the detection of neutrophils in tissue possible, since it detects the oxidative burst of neutrophils, which can be increased due to more neutrophils at site or due to them being more activated. Skin MPO readings of C57BL/6 were found to be higher at 24 hours post-burn compared to the readings in LBPko, rising in parallel with knockouts as time progressed. Since, as indicated before, cellular immune reactions seen in leukocyte subsets being attracted to the site of inflammation follows gene expression with a delay of one day, we started our MPO analysis at 24 hours following burn. It is possible that the time point we have chosen to begin analyzing neutrophils intradermally was too late and only reflected the kinetics in wt mice adequately, thus possibly giving an incomplete picture. To our knowledge, neutrophil behavior with reference to host immunity in LBPko mice having sustained burns has not been studied yet. To obtain a better picture of this early kinetics, future studies need to focus on neutrophils in LBP-knockout mice and their possible intradermal presence at earlier time points post-burn.

With regard to the peripheral blood pool of leukocytes, we found that the total number of leukocytes was higher in knockouts at 24 hours after burn. At 96 hours, knockouts seemed to have almost 4-times as many leukocytes in their blood as wild-types. This WBC

increase found in LBPko mice was attributable mainly to lymphocyte increase, and indicates a profoundly different strategy of post-burn inflammation response. This diverse cellular immune reaction between knockouts and wt has potential effects on coping with infections like those in our burn wound model. In order to gain more information, we stepped from a defined infection protocol, as conducted in our previous experiments, to a natural course of bacterial re-colonialisation. Regularly, burn wounds are found to be sterile after the injury and become re-colonialized over time from the surrounding skin flora. After 6 days we found that the majority of the bacteria encountered in both groups were Gram-positive, which corresponds with naturally occurring, surrounding skin flora. Unexpectedly, 20.6-fold more Gram-positive bacteria were found on the skin of wild type mice. The clearance of Gram-positive bacteria involves the protein lipoteichoic acid (LTA) followed by binding to the TLR2 system in contrast of binding to the TLR4 complex, which is used by the LBP/LPS complex of Gram-negative bacteria (42, 43), as previously shown. One would therefore expect, that Gram-positive bacterial clearance was independent of LBP-ablation as seen in LBPko animals, in contrast to the observed 20-fold less Gram-positive bacteria. Possibly, 'compensatory' immunological mechanisms leading to reduced Gram-positive bacteria in burn wounds of LBPko animals account for this observation and need further exploration. Within our experiments, we possibly revealed support for Golenbock´s theory that a mechanism exists that compensates for missing LBP (27, 44), which has also been proposed by this very group, who created the LBPko mouse model in the first place.

Conclusion

LBP-deficiency in knockout mice used in a standardized burn wound model led to profoundly changed peripheral leukocyte and leukocyte subset numbers, as well as IL-6 and GRO-1intradermal mRNA levels when compared to wt mice over a time course of 96 hours. At the burn site, the intradermal accumulation of neutrophils revealed lower readings in LBPko mice, indicating lower numbers or less activated neutrophils in knockout animals. Furthermore, we observed less Gram-positive bacteria on the skin of knockout mice as a result of natural re-colonialisation. The complex immunological interactions of leukocyte subsets and the complexity of spatiotemporal cytokine expression locally at the site of the wound, all influencing topical Gram-positive as well as Gram-negative bacterial growth, justifies further studies focusing on the compensatory mechanisms prevailing in infections in LBPko mice.

References

1. Pruitt BA, Jr., McManus AT. The changing epidemiology of infection in burn patients. *World J Surg* 1992;16:57-67.
2. Azzopardi EA, Azzopardi SM, Boyce DE, et al. Emerging gram-negative infections in burn wounds. *J Burn Care Res* 2011;32:570-576.
3. Branski LK, Al-Mousawi A, Rivero H, et al. Emerging infections in burns. *Surg Infect (Larchmt)* 2009;10:389-397.
4. Church D, Elsayed S, Reid O, et al. Burn wound infections. *Clin Microbiol Rev* 2006;19:403-434.
5. Sarabahi S, Tiwari VK, Arora S, et al. Changing pattern of fungal infection in burn patients. *Burns [Epub ahead of print]* 2011.
6. Mason AD, Jr., McManus AT, Pruitt BA, Jr. Association of burn mortality and bacteremia. A 25-year review. *Arch Surg* 1986;121:1027-1031.
7. Galeiras R, Lorente JA, Pertega S, et al. A model for predicting mortality among critically ill burn victims. *Burns* 2009;35:201-209.
8. D'Avignon LC, Hogan BK, Murray CK, et al. Contribution of bacterial and viral infections to attributable mortality in patients with severe burns: an autopsy series. *Burns* 2010;36:773-779.
9. Cartotto RC, Macdonald DB, Wasan SM. Acute bacterial endocarditis following burns: case report and review. *Burns* 1998;24:369-373.
10. Mahar P, Padiglione AA, Cleland H, et al. Pseudomonas aeruginosa bacteraemia in burns patients: Risk factors and outcomes. *Burns* 2010;36:1228-1233.
11. Lipovy B, Rihova H, Hanslianova M, et al. Prevalence and resistance of Pseudomonas aeruginosa in severely burned patients: a 10-year retrospective study. *Acta Chir Plast* 2010;52:39-43.
12. Revathi G, Puri J, Jain BK. Bacteriology of burns. *Burns* 1998;24:347-349.
13. Tobias PS, Mathison J, Mintz D, et al. Participation of lipopolysaccharide-binding protein in lipopolysaccharide-dependent macrophage activation. *Am J Respir Cell Mol Biol* 1992;7:239-245.
14. Su GL, Freeswick PD, Geller DA, et al. Molecular cloning, characterization, and tissue distribution of rat lipopolysaccharide binding protein. Evidence for extrahepatic expression. *J Immunol* 1994;153:743-752.
15. Wright SD, Ramos RA, Tobias PS, et al. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* 1990;249:1431-1433.
16. Yang RB, Mark MR, Gray A, et al. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. *Nature* 1998;395:284-288.
17. Poltorak A, He X, Smirnova I, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 1998;282:2085-2088.
18. Su GL, Klein RD, Aminlari A, et al. Kupffer cell activation by lipopolysaccharide in rats: role for lipopolysaccharide binding protein and toll-like receptor 4. *Hepatology* 2000;31:932-936.
19. Klein RD, Su GL, Aminlari A, et al. Skin lipopolysaccharide-binding protein and IL-1beta production after thermal injury. *J Burn Care Rehabil* 2000;21:345-352.
20. Huber NL, Bailey SR, Schuster RM, et al. Remote thermal injury increases LPS-induced intestinal IL-6 production. *J Surg Res* 2010;160:190-195.

21. Finnerty CC, Herndon DN, Przkora R, et al. Cytokine expression profile over time in severely burned pediatric patients. *Shock* 2006;26:13-19.
22. Cavaillon JM, Annane D. Compartmentalization of the inflammatory response in sepsis and SIRS. *J Endotoxin Res* 2006;12:151-170.
23. Gosain A, Gamelli RL. A primer in cytokines. *J Burn Care Rehabil* 2005;26:7-12.
24. Summer GJ, Romero-Sandoval EA, Bogen O, et al. Proinflammatory cytokines mediating burn-injury pain. *Pain* 2008;135:98-107.
25. Calle Y, Burns S, Thrasher AJ, et al. The leukocyte podosome. *Eur J Cell Biol* 2006;85:151-157.
26. Tyler MP, Watts AM, Perry ME, et al. Dermal cellular inflammation in burns. an insight into the function of dermal microvascular anatomy. *Burns* 2001;27:433-438.
27. Wurfel MM, Monks BG, Ingalls RR, et al. Targeted deletion of the lipopolysaccharide (LPS)-binding protein gene leads to profound suppression of LPS responses ex vivo, whereas in vivo responses remain intact. *J Exp Med* 1997;186:2051-2056.
28. Gilpin DA. Calculation of a new Meeh constant and experimental determination of burn size. *Burns* 1996;22:607-611.
29. Su GL, Hoesel LM, Bayliss J, et al. Lipopolysaccharide binding protein inhibitory peptide protects against acetaminophen-induced hepatotoxicity. *Am J Physiol Gastrointest Liver Physiol* 2010;299:G1319-1325.
30. Goldblum SE, Wu KM, Jay M. Lung myeloperoxidase as a measure of pulmonary leukostasis in rabbits. *J Appl Physiol* 1985;59:1978-1985.
31. DeForge LE, Takeuchi E, Nguyen DT, et al. Immunological priming attenuates the in vivo pathophysiological response to lipopolysaccharide. Comparison of cytokine production, tissue injury, and lethality in complete Freund's adjuvant-primed mice and in unprimed mice. *Am J Pathol* 1994;144:599-611.
32. Cole J, Tsou R, Wallace K, et al. Early gene expression profile of human skin to injury using high-density cDNA microarrays. *Wound Repair Regen* 2001;9:360-370.
33. Theilgaard-Monch K, Knudsen S, Follin P, et al. The transcriptional activation program of human neutrophils in skin lesions supports their important role in wound healing. *J Immunol* 2004;172:7684-7693.
34. Li X, Mohan S, Gu W, et al. Analysis of gene expression in the wound repair/regeneration process. *Mamm Genome* 2001;12:52-59.
35. Feezor RJ, Paddock HN, Baker HV, et al. Temporal patterns of gene expression in murine cutaneous burn wound healing. *Physiol Genomics* 2004;16:341-348.
36. Ravat F, Payre J, Peslages P, et al. [Burn: An inflammatory process]. *Pathol Biol (Paris)* 2011;59:e63-72.
37. Steinstraesser L, Burkhard O, Fan MH, et al. Burn wounds infected with Pseudomonas aeruginosa triggers weight loss in rats. *BMC Surg* 2005;5:19.
38. Shubin NJ, Monaghan SF, Ayala A. Anti-inflammatory mechanisms of sepsis. *Contrib Microbiol* 2011;17:108-124.
39. Lederer JA, Brownstein BH, Lopez MC, et al. Comparison of longitudinal leukocyte gene expression after burn injury or trauma-hemorrhage in mice. *Physiol Genomics* 2008;32:299-310.

40. Bozic CR, Gerard NP, von Uexkull-Guldenband C, et al. The murine interleukin 8 type B receptor homologue and its ligands. Expression and biological characterization. *J Biol Chem* 1994;269:29355-29358.
41. Lee J, Cacalano G, Camerato T, et al. Chemokine binding and activities mediated by the mouse IL-8 receptor. *J Immunol* 1995;155:2158-2164.
42. Kirschning CJ, Bauer S. Toll-like receptors: cellular signal transducers for exogenous molecular patterns causing immune responses. *Int J Med Microbiol* 2001;291:251-260.
43. Mitchell JA, Paul-Clark MJ, Clarke GW, et al. Critical role of toll-like receptors and nucleotide oligomerisation domain in the regulation of health and disease. *J Endocrinol* 2007;193:323-330.
44. Fenton MJ, Golenbock DT. LPS-binding proteins and receptors. *J Leukoc Biol* 1998;64:25-32.

Chapter 5

Antimikrobielle Peptide und Fibrinkleber in Verbrennungen

5

Ein Gemisch aus einem antimikrobielle Peptid und Fibrinkleber behält biologische Potenz *in vitro* und gegen multiresistente *Pseudomonas aeruginosa* Bakterien in Verbrennungen der Tiefe IIb *in vivo*

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A mixture of antimicrobial peptides and fibrin glue in treatment of partial-thickness burn wounds; Lahoda LU, Wang SC, Vogt PM. Chirurg. 2006 Mar;77(3):251-6

Zusammenfassung:

Hintergrund:
Antimikrobielle Peptide sind natürlich vorkommende kationische Peptidmoleküle. Die erste Verteidigungslinie der Verbrennungswunde stellt das angeborene Immunsystem dar, deren Bestandteile diese Peptide sind. Um die topische Anwendbarkeit in infizierten Verbrennungswunden zu vereinfachen wurde die Wirksamkeit in Fibrinkleber *in vivo* und *in vitro* getestet.

Material und Methoden:
Nach *in vitro* Testung erhielten 15 männliche Sprague-Dawley Ratten eine tief-zweitgradige Verbrennung und wurden mit multiresistenten *Pseudomonas aeruginosa* infiziert und mit PG-1 (100ug/ml, n=5), Fibrinkleber (n=5) oder einem Gemisch aus beiden (n=5) topisch behandelt, die Wirkung wurde zuvor durch einen Radial-Diffsusionsassay bestätigt. 24 Stunden später wurde die verbrannte und infizierte Haut gewonnen und die Bakterienanzahl pro Gramm Haut bestimmt.

Ergebnisse:
In vitro ließ sich die biologische Aktivität bestätigen. Die Gruppe aus entweder PG-1 oder Fibrinkleber zeigte *in vivo* keine signifikanten Unterschiede in der Bakterienanzahl, hingegen ließ sich in der Gruppe des Gemisches eine signifikante antibakterielle Wirkung nachweisen ($p<0,04$ und $p<0,01$).

Schlußfolgerugen:
Eine Mischung aus dem antimikrobiellen Peptid PG-1 und Fibrinkleber reduziert die Bakterienzahl eines definierten Infektes einer IIb-Verbrennung *in vivo* signifikant im Vergleich zu den Kontrollgruppen.

Schlüsselwörter:
Verbrennung, angeborenes Immunsystem, Infektion, antimikrobielle Peptide, Fibrinkleber

Abstract:

Background:
Antimicrobial peptides are naturally occurring cationic peptide. The first line of defense in infected burns is the innate immune system of which antimicrobial peptides are essential parts. To facilitate their topical use in infected partial thickness burns, the efficacy of a mixture of the antimicrobial peptide PG-1 with fibrin glue *in vitro* and *in vivo* was tested.

Methods:
After *in vitro* tests, 15 male Sprague-Dawley rats received partial thickness burns; following, the wounds were infected with multiresistant *Pseudomonas aeruginosa*. Animals received either the antimicrobial peptide Protegrin-1 (PG-1) (100μg/ml, n=5), fibrin glue (n=5) or a mixture of both (n=5) topically, efficacy was previously proven by radial diffusion assay. After 24 hours, the infected and burned skin was harvested and quantitative bacterial counts per Gram skin performed.

Results:
The biologic effect of the peptide was confirmed *in vitro*. The PG-1 group and the fibrin glue group did not show significant differences in bacterial numbers, whereas the mixture group showed significant reduction in *Pseudomonas in vivo* ($p<0.04$ and $p<0.01$).

Conclusion:
A mixture of an antimicrobial peptide and commercially available fibrin glue is capable of significantly reducing bacteria in infected partial thickness burns *in vivo* compared to their controls.

Key words:
Burns, innate immune system, infection, antimicrobial peptides, fibrin glue

Einleitung:

Infizierte Brandwunden stellen eine Quelle hoher Morbidität und Mortalität dar. Die fehlende Schutzfunktion verbrannter Haut erleichtert eine rasche Infektion aus bakterieller Kolonisation. Unbehandelt kann je nach Keimspektrum, Infektdauer und Resistenz des betroffenen Organismus, je nach Virulenz, Art und Ausdehnung der Verbrennung ein „systemic inflammatory response syndrome" (SIRS), Multiorganversagen und Sepsis folgen. Aus einem lokalen Wundproblem wird ein den gesamten Organismus bedrohendes Krankheitsbild. Ein Fundament der Therapie des Schwerbrandverletzten stellt die lokale antibakterielle Therapie dar. Topische Antibiotika sind zwar effektiv, führen jedoch unter Umständen zur Resistenzentwicklung und/oder der Allergisierung.

Eine neue Substanzgruppe, antimikrobielle Peptide (AMPs), sind natürlich vorkommende Peptidmoleküle mit sehr breiter Wirkung gegen Gram-positive und Gram-negative Bakterien, Viren (mit Lipidhüllen) und Pilzen. Sie werden als alte evolutionäre Waffen [1] bezeichnet, die eine „Achillesferse" der attackierenden Einzeller angreifen, nämlich deren Zellmembran, die sich prinzipiell strukturell von der der Mehrzeller unterscheidet. AMPs werden bei Tieren, Pflanzen und dem Menschen beschrieben, sind seit ca. 70 Jahren bekannt und werden nicht nur in der medizinischen Forschung, sondern auch in der Tabakindustrie und im Kartoffelanbau zur Ertragssteigerung eingesetzt [2].

Verbrennungswunden weisen nicht nur Eintrittspforten für Mikroorganismen auf, sondern über sie gehen Flüssigkeit und Eiweiße verloren. Einen kutanen Wundabschluß im Rahmen der Heilung bewirkt Fibrin und die stufenweise ablaufenden Phasen der Gerinnung. Das Rationale des Einsatzes von Fibrinkleber besteht darin, das Einsprossen von Immuneffektorzellen und epithelbildenden Zellen zu erleichtern und die frühen Stufen der Wundheilung in Gang zu setzen. Allerdings bietet natives Fibrin ein Nährmedium für Bakterienwachstum, dem es entgegenzuwirken gilt. Die naheliegende Idee, Antibiotika zum Fibrinkleber zu mischen und dadurch einerseits die Angriffsfläche des Kleber-Antibiotikagemisches zu vergrößern sowie die Kontaktdauer zu verlängern und andererseits die unerwünschten Nebenwirkungen zu reduzieren um gleichzeitig lokale antimikrobielle Effekte zu erzielen, wurde bereits unter verschiedenen Gesichtspunkten mehrerer Forschergruppen untersucht und publiziert [3-5]. Im aktuellen innovativen Ansatz wird Fibrinkleber als Vektor zur Applikation der AMPs eingesetzt. Daten zu diesem Vorhaben liegen derzeit noch nicht vor, technische Details sind noch nicht etabliert.

Ziel der vorliegenden Studie war daher die Frage, ob antimikrobielle Peptide gemischt mit Fibrinkleber potente Effektoren der Bakterienhemmung in infizierten Brandwunden sein können.

Material und Methoden:

In vitro Experimente:

Protegrin-1:

Das unsererseits verwendete antimikrobielle Peptid Protegrin-1 (PG-1), ursprünglich aus Schweineneutrophilen isoliert, wurde entsprechend der publizierten Strukturformel [6] (*Abbildung 1*) aus 18 Aminosäuren und 2 Disulfidbrücken kommerziell in Auftrag gegeben und hergestellt (BioSynthesis, Lewisville TX, USA). Das gelieferte lyophilisierte Peptid wurde entsprechend den Vorgaben des Herstellers gelöst und dadurch in die biologisch aktive Form transferiert, die Effektivität des Proteins wurde durch Radial-Diffusions-Assays getestet [7].

Radial-Diffusions-Assay:

Mittels eines Radial Diffusion Assay (bakterieller Wachstumshemmtest gegen antibakteriell wirksame Substanzen) wurde die *in vitro*-Aktivität des neuen Gemisches aus Protegrin-1 und dem Gewebekleber überprüft. Zusammengefasst bestand die erste der zwei Schichten aus 1%-Agarose (Sigma Chemical, St. Louis, MO USA) und 0,03% Trypticase Soy Broth mit 10mM Natriumphosphat und 100mM NaCl bei einem pH von 7,4. Die nachfolgende Abdeckschicht bestand aus 6%igem Tryticase Soy Broth und 1%iger Agarose in phosphatgepuffertem Kochsalz. Als Kontrollorganismus wurde hier *E.coli* (eingestellt auf 200µl mit $4x10^7$ Bakterien) mit 10ml des ersten Mediums gemischt und in 10cm Petrischalen ausgegossen. Nach dem Erstarren wurden 3mm-Löcher in das Zentrum des Agars gestanzt und mit 30µl der jeweiligen Testsubstanzen gefüllt (*Tabelle 1*). Das Sealer Protein, der Fibrinolyse-Inhibitor, Thrombin und Calziumchlorid wurden entsprechend den Vorgaben des Erzeugers (Baxter Healthcare, Glendale, CA USA) gemischt und kurzfristig bei 37°C gehalten, bis die Komponenten verwendet wurden. Gentamyzin mit einer Konzentration von 3,16 µg/ml wurde als Positiv-Kontrolle desselben Volumens im zentralen Stanzloch appliziert. Nachfolgend wurden alle Platten denselben Bedingungen

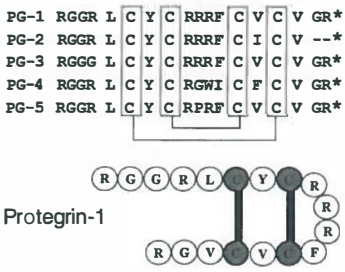

Abbildung 1. Primäre und Sekundäre Struktur von Protegrin-1 (PG-1), mit 2 Disulfid-Brücken, Molekulargewicht von 2157 Da (Dalton)

Konzentration (Mischung von Kleberprotein + Fibrinogen / PG1)	**Kleberprotein Lösung** (+Aprotinin)	**Thrombin Lösung** (+ $CaCl_2$)	**PG-1,** 100 µg/ml	**Volumen** pro Well
1:1	7,5 µl	7,5 µl	15 µl	30 µl
1:2	5 µl	5 µl	20 µl	30 µl
2:1	10 µl	10 µl	10 µl	30 µl

Tabelle 1. Aufbau der *in vitro*-Studie: Konzentrationen und Verhältnisse von PG-1 und dem Fibrinklebergemisch mit seinen Komponenten

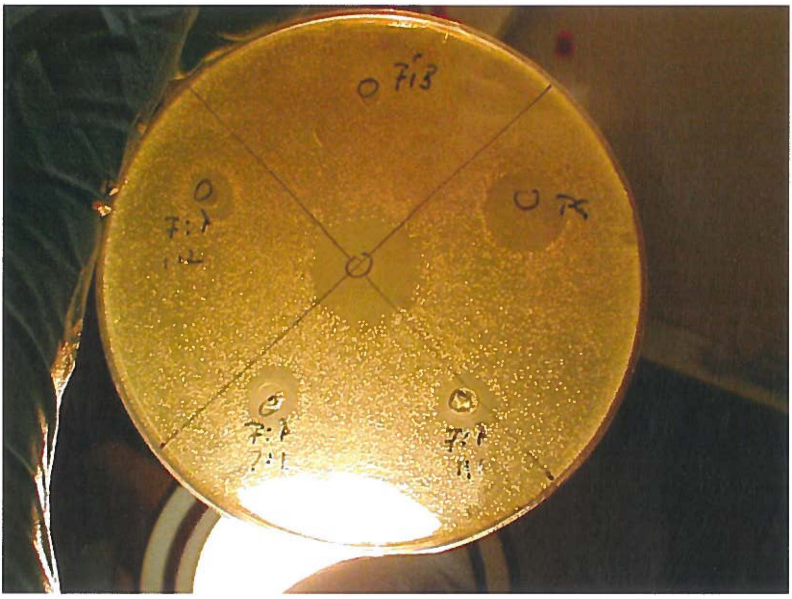

Abbildung 2. Radial Diffusion Assay (Durchlichtbetrachtung): Hemmung von Bakterienwachstum durch PG-1 („PG" und „P"), Fibrinkleber („FIB" und „F") und verschiedenen Mischungsverhältnissen (F:P) der beiden Komponenten. Zentral die Positiv-Kontrolle durch Gentamyzin

ausgesetzt und bei 37°C für 3 Stunden inkubiert, danach folgte das Abdecken durch den zweiten, nährstoffreichen Agar (10ml) als overlay. Nach 18 Stunden Inkubation in einem Brutschrank bei 37°C, wurden die Platten analysiert. Die klaren Zonen um die Stanzlöcher lassen auf gehemmtes Bakterienwachstum schließen (positives Ergebnis), wohingegen der restliche Agar trüb, da mit Bakterien durchsetzt, erscheint. Der Durchmesser diesen hellen Zonen wurde mit der Positiv-Kontrolle (Gentamyzin) derselben Platte korreliert, um ein Maß der antibakteriellen Aktivität zu erhalten (*Abbildung 2*).

Tisseel®, Tissucol® (Baxter, IL, USA):
Entsprechend dem Protokoll des Herstellers wurden die insgesamt 4 Komponenten des kommerziell erhältlichen Gewebeklebers auf 37 Grad Celsius erwärmt und zu zwei Komponenten vermischt (Fibrinogen und Thrombin). Bis zur endgültigen Mischung beider Teile zum aktiven Fibrin, wurde die o.g. Temperatur für wenige Minuten beibehalten und in fallender Konzentration erfolgte die Mischung des vorbereiteten Protegrin-1 mit der Thrombinkomponente in entsprechenden Verhältnissen (*s. Tabelle 1*). Die resultierenden Mischungen wurden erst im ausgestochenen Stanzdefekt des Agars zusammengeführt und somit die Fibrinbildung mit PG-1-Inhalt initiiert. *Abbildung 2* zeigt den Versuchsansatz in unterschiedlichen Mischungsverhältnissen der PG-1- und Fibrinkleber-Komponenten auf den Radial-Diffusionsplatten. Das dabei ermittelte, für die weiteren Versuche adäquate Mischungsverhältnis wurde für die Anwendung in den *in vivo* Experimenten eingesetzt.

In vivo Experimente:

Tierexperiment:
Für die *in vitro* Experimente wurden männliche, ausgewachsene Sprague-Dawley-Ratten (n=15) mit einem Durchschnittsgewicht von 250 Gramm einer 15%igen Verbrennung/ Verbrühung ihrer Körperoberfläche unterzogen (Tiefe IIb) und in 3 gleiche Gruppen randomisiert. Die Verbrennung wurden entsprechend den Vorgaben des National Institutes of Health (NIH) und der Tierexperimentellen Einrichtung der University of Michigan (ULAM, UCUCA) für kleine Nager in Allgemeinnarkose mit gewichtsadaptierter Gabe von Ketamin und Xylazin intraperitoneal durchgeführt und von der Ethikkomission genehmigt. Postoperativ erfolgte die gewichtsadaptierte Analgesie mit Bupivacain (Buprenex®) alle 6-8 Stunden. Die Tiere wurden bis zur völligen Mobilität überwacht, der Flüssigkeitsersatz erfolgte durch die subkutane Applikation von physiologischer Kochsalzlösung. Die Ermittlung der Verbrennungsfläche ergab sich aus der Formel von Meeh [8]. Die Wunden wurden sofort nach der Verbrühung unter sterilen Kautelen mittels $1x10^6$ *Pseudomonas aeruginosa* inokuliert. Die Pseudomonas-Bakterien waren durch ein Bakteriogramm vor den Experimenten als multiresistent (unter anderem Silvadene®/Silbersulfadiazine) eingestuft worden.
In 3 Gruppen (je 5 Tiere) wurde entweder topisch ein Gemisch aus Fibrinkleber und Protegrin-1 (PG-1) (50 Volumen % PG-1, Konzentration des PG-1 100µg/ml, 50 Volumen % der Thrombin/Fibrinogen-Mischung, 2,5ml pro Applikation), oder ungemischtes, flüssiges PG-1 (2,5 ml, Konzentration: 100µg/ml, Kontrollgruppe), oder 2,5 ml des Fibrinklebergemisches (negative Kontrolle) topisch appliziert. Pro Untersuchungsgruppe wurde die Mischung aus Protegrin mit dem Kleber und die einzelnen Komponenten, zusammen mit den Bakterien unter sterilen okklusiven Verbänden aufgebracht, um Kreuz-Kontaminati-

onen für die Zeit des Versuches zu vermeiden (*Abbildung 3*). Es wurde hierzu ein steriler Tupfer auf das Verbrennungsareal mit den unterschiedlichen Komponenten aufgebracht, durch eine Klebefolie (ähnlich einer OP-Folie) verschlossen und eine Manipulation durch das Tier ausgeschlossen, indem selbsthaftendes, elastisches Verbandsmaterial überwickelt wurde, das die Mobilität zur Reinigung und Nahrungsaufnahme der Tiere nicht beeinträchtigte.

Nach einer Inkubation über 24 Stunden wurden Hautbiopsien entnommen, gewogen, homogenisiert und als Verdünnungsreihe in Triplets auf Schafblutagarplatten aufgebracht und über Nacht inkubiert. Am folgenden Tag wurden die Pseudomonaskulturen Untersucher-geblindet ausgezählt. Die Bestimmung des Pseudomonaswachstums erfolgte morphologisch anhand der Kulturen. Daraus wurden die Bakterienzahlen pro Gramm Haut im Vergleich der drei Gruppen ermittelt (*Abbildung 4*).

Abbildung 3. Betäubte Sprague-Dawley Ratte mit okklusivem Verband entsprechend der Versuchsanordnung unter selbstklebendem und elastischem Schutzverband gegen die Manipulation durch das Tier; freie Mobilität mit dem Verband bleibt erhalten

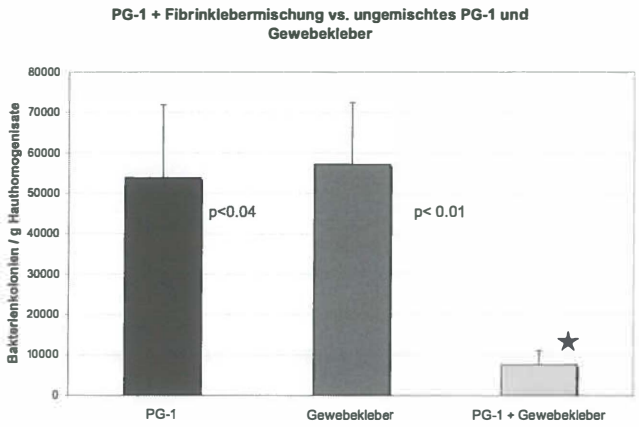

Abbildung 4. Reduktion der Keimzahlen in *Pseudomonas*-infizierten Verbrennungswunden (Hauthomogenisate). Signifikanter antimikrobieller Effekt bei Vermischung von PG-1 mit Fibrinkleber gegenüber den ungemischten Komponenten ($p<0{,}04$ und $p<0{,}01$)

Ergebnisse:

***In vitro* Experimente:**

Im Radial-Diffusionsassay zeigte sich eine von der jeweiligen PG-1 Menge abhängige antimikrobielle Wirkung des ungemischten PG-1 und der PG-1-Kleber-Gemische (*Abbildung 2*). Die antimikrobielle Aktivität des PG-1 wurde durch das Beimischen von Fibrinkleber nicht inaktiviert, sondern proportional zum Verdünnungsvolumen reduziert. Das in Mischung eingebrachte PG-1 zeigte abhängig vom Volumenanteil eine Wachstumshemmung der in Agar wachsenden *E.coli*-Bakterien, erkennbar am „Hemmhof". Die Zone der Wachstumshemmung (mit Gentamycin als 100%) zeigte eine Verminderung auf 50,7% mit Protegrin-1 allein, auf 29,4% mit dem Peptid-Fibrinklebergemisch (Mischungsverhältnis 1:1), auf 26,3% mit einem Mischungsverhältnis von 1:2 Fibrinkleber zu PG-1 und auf 16,5% mit dem Mischungsverhältnis 2:1 Kleber zu PG-1 (*Tabelle 2*). Die positive und ne-

Gentamyzin	100%
PG-1	50,7%
Kleber-PG1 1:1	29,4%
Mischung Kleber-PG1 1:2	26,3%
Mischung Kleber-PG1 2:1	16,5%

Tabelle 2. Hemmungswirkung auf das Bakterienwachstum der experimentellen Gruppen *in vitro* in Relation zur Positivkontrolle im Radialdiffusionsassay (zu *Abbildung 2*) (Positiv-Kontrolle: Gentamyzin, Negativ-Kontrolle: Kleber, Gruppen mit unterschiedlichem Volumengehalt von Kleber zu PG-1)

gative Kontrolle sind als „Proof of concept" des wissenschaftlichen Ansatzes zu werten. Es zeigte sich eine maximale bakterielle Inhibition durch das zentral verbrachte Gentamyzin und keine bakterielle Hemmung durch den reinen Fibrinkleber. Die *in vitro*-Experimente des Radial-Diffusion-Assay bestätigten somit unseren wissenschaftlichen Ansatz, dass die Mischung aus Gewebekleber und Protegrin-1 ebenso wie das antimikrobielle Peptid allein eine antibakterielle Wirksamkeit auch gegen einen resistenten *Pseudomonas aeruginosa* Stamm besitzt.

***In vivo* Experimente:**

Die Bakterienzahlen in den *in vivo* Experimenten zeigten keine signifikanten Unterschiede im Vergleich der ungemischten Protegrin-1 und Gewebekleber-Gruppen (*Abbildung 4*) nach der Übernacht-Inkubation (Gruppe PG-1: 53908 ± 18020 (Kolonien/Gramm Hauthomogenisat), $p<0,04$; Gruppe Fibrinkleber: 57219 ± 15273, $p<0,01$; Gruppe PG-1 plus Fibrinkleber: 7621 ± 3495). Die Tabelle zeigt die Anzahl der Bakterienkolonien (Mittel aus dreifachem Ansatz) pro Gramm Haut im Vergleich der drei gebildeten Gruppen. Die Tiere, welche die Mischung aus dem Gewebekleber und dem PG-1 erhalten hatte, zeigten eine signifikante Verminderung der Zahl der Bakterien verglichen mit den beiden Kontrollgruppen ($p<0,04$ und $p<0,01$). Kein Tier verstarb während des Experimentes (s. *Abb. 4;* Statistik: „unpaired means of comparison", StatView®, SAS Inc. USA). Im Vergleich beider Kontrollgruppen mit der Mischung aus Kleber und dem antimikrobiellen Peptid, ergab sich eine Reduktion der Bakterien auf ca. ein Fünftel. In unserer Analyse wurde keine Kreuz-Kontamination mit anderen Bakterien als *Pseudomonaden* gesehen.

Diskussion:

Antimikrobielle Peptide sind bereits seit ungefähr 70 Jahren im Tier- und Pflanzenreich bekannt [9]. So besitzt selbst die *Drosophila melanogaster* die Fähigkeit zu ihrer Produktion. Gewöhnlich sind diese Peptide ungefähr 100 Aminosäuren lang und werden von individuellen Genen codiert, die eine Intron-Exon-Struktur aufweisen [10]. Bis heute kennt man über 500 solcher antimikrobieller Peptide, wobei regelmäßig neue Beschreibungen publiziert werden (http://www.bbcm.univ.trieste.it/~tossi/antimic.html). Charakteristischerweise bestehen sie aus hydrophilen und hydrophoben dreidimensionalen Strukturen, die es ihnen ermöglichen, durch Einbau in die bekannten Lipidschichten der Zellwände „Poren" zu formen und durch einen Austausch von extra- und intrazellulären Ionen zum Zelltod zu führen (*Shai-Matsuzaki-Huang Modell*) [11, 12]. Aktuell wird diskutiert, dass der Zellwandaufbau (die Mischung der unterschiedlichen Lipide) einen essentiellen Einfluß auf die Effizienz dieser Peptide spielt und zur Unterscheidung zwischen „körpereigen" und „körperfremd", im Vergleich der Mehrzeller zu den Einzellern beiträgt. Lymphozyten, Endothelzellen und Keratinozyten produzieren AMPs und geben dadurch deutliche Hinweise auf deren Wichtigkeit zum Schutz der endothelialen wie der epithelialen Oberflächen der Lunge, der Haut und des Gastrointestinaltraktes [13, 14]. Im Menschen finden sich *Defensine*, *Cathelizidine* (wie Protegrin-1, LL37 usw.) und *Histatine* [10]. Wenn man nach einem „Mangelsyndrom" der AMPs als Ursache von Erkrankungen oder möglichen Hinweisen auf die Pathogenese dieser Erkrankungen sucht, so ergeben sich Verbindungen zum Chediak-Higashi-Syndrom, zur zystischen Fibrose und Einflüsse bei genereller Immundefizienz [2, 15]. Diesem postulierten Mangel steht die genetisch nachweisbare „up-regulation" durch die Anwesenheit und Stimulation von Lipopolysaccharid, LPS (Synonym für „Endotoxin" und entspricht dem wesentlichen Bestandteil von Gram-negativen Bakterienwänden), Interleukin 6 und retinoischer Säure gegenüber.

Protegrin-1 (PG-1, *Abbildung 1*), ein Vertreter der antimikrobiellen Peptide das ursprünglich aus Schweine-Neutrophilen isoliert wurde, konnte in Vorexperimenten unseres Labors die bakterielle Infektionsrate in einem Rattenverbrennungsmodell deutlich reduzieren [16]. Andere Untersucher konnten in ihren Tiermodellen zeigen, dass sich einerseits die Überlebensrate durch die topische Anwendung der Peptide in Verbrennungen dramatisch steigern ließ [17], in einem anderen Fall wurde die Menge eines Oberflächenpeptids (humanes Beta-Defensin) in brochoalveolären Lavagen von Patienten mit Verbrennungen im Vergleich zu einer Kontrollgruppe untersucht [18]. Auch hier zeigten sich erhöhte Werte bei Verbrennungspatienten, ein Hinweis für die vermehrte Produktion durch das Verbrennungstrauma. In einem weiteren Versuch konnte gezeigt werden, dass in Verbrennungsarealen verminderte Konzentrationen der Peptide (Defensine) perifokal vor-

lagen und korrelierten [19], bei zweitgradigen Verbrennungen verlagerten sich die Produktionsstätten der Peptide aus der Dermis in deren Anhängsel wie Haarfollikel oder Schweißdrüsen. Die Wirkung der Peptide ist demnach nicht nur einem einzigen Peptid zuzuordnen. Im Gegensatz zu unserem Verbrennungsmodell verwenden die meisten Untersucher derzeit drittgradige Verbrennungen, ausgenommen Milner et al [19].
Verbrennungen bis zur Tiefe IIB sind Gegenstand unseres Interesses, da sich die Frage stellt, ob bei tiefdermalen Verbrennungen der Organismus in der Lage ist, das infizierte Wundmilieu bei verlorengegangener Schutzschicht der Haut durch die ortständige Abwehr des angeborenen Immunsystems zu kompensieren und wie die lokalen Defensivstrategien gegen eindringende Krankheitserreger aussehen. In mehreren Versuchen der klinischen Umsetzung der attraktiven Potenz antimikrobieller Peptide werden zur Zeit Phase II und III Tests durchgeführt [2], die sich allerdings auf Themen wie den diabetischen Fuß und dessen komplizierende Ulzerationen, sowie die orale Mukositis [20] und das Beschichten von intravenösen Dauerkathetern konzentrieren.
Die Erforschung im Rahmen von Verbrennungen wird derzeit nur von wenigen internationalen Forschergruppen vorgenommen. Entsprechend unserem Forschungs-schwerpunkt, wurde nach Etablierung eines standardisierten Verbrennungsmodelles an kleinen Nagern, Untersuchungen zur Wirkung der lokale Applikation von Protegrin-1 (PG-1) in infizierten Verbrennungen der Tiefe IIB durchgeführt. Es sollte die biologische Aktivität und Wirkung von einem Gemisch aus Fibrinkleber mit PG-1 gegen einen multiresistenten Stamm *Pseudomonas aeruginosa* nach Vorversuchen *in vitro* und *in vivo* bestimmt werden. Das in flüssiger Form vorliegende aktive Peptid stellte eine Herausforderung im Handling dar. Ein Lösungsansatz ergab sich durch die Kombination mit Gewebekleber, um nunmehr einfachere Anwendbarkeit, die Problematik der Applikation auf unebenen Oberflächen und die gewünschte topische antibakterielle Aktivität möglichst optimal verbinden zu können. In aus der Literatur bekannten Studien war die Umsetzung der Idee topischer antibakteriell wirksamer Substanzen in mehreren Studien vorbeschrieben [3-5, 21-23]. Nach einer Serie von *in vitro*-Versuchen mit unterschiedlichen Konzentrations- und Mischungsverhältnissen, in enger Anlehnung an die Richtlinien zur Verwendung des Gewebeklebers, zeigte sich die mögliche klinische Anwendbarkeit, indem das verwendete Peptid nicht durch den Kleber in seiner antibakteriellen Wirkung neutralisiert wurde. Zudem erleichterte sich das Aufbringen des Protegrins *in vivo* durch den Kleber.
Die Ergebnisse unserer Tierstudien erklären sich auch durch die Art der Applikation einerseits als flüssiges Medium des puren Peptids, andererseits des Klebergemisches plus Peptid. Erwartungsgemäß sollten die Bakterien durch das „pure", unvermischte Protegrin-1 deutlich reduziert werden, allerdings ist die Zahl der Bakterienkolonien ähnlich hoch wie in der Gruppe der nur mit dem Kleber „behandelten" Tiere. Eine mögliche Erklärung ist die zunehmende Kontaktzeit des Peptids zusammen mit dem Kleber, der in nur flüssiger Form leicht von der Rattenhaut abtropft und durch Aufnahme in den sterilen

Verband an Wirkung zu verlieren scheint. Im Gegensatz dazu ist durch das Mischen mit dem Kleber ein Film aus Kleber-Peptid-Gemisch auf der Haut präsent, der dort seine antibakterielle Effizienz lokal verbreiten kann, ein Effekt der durch den Kleber allein nicht auftritt. Es ist bekannt, dass die Peptide ca. 6 Stunden Wirksamkeit aufweisen, bevor das bakterielle Wachstum wieder in Gang kommt und die zuvor erzielte Keimzahlreduktion bald wieder zunichte macht. Der Organismus schafft dies durch ständige Neuproduktion und Mischung verschiedener Klassen von AMPs im klinischen Bedarfsfall. In unseren Experimenten wurde einmalig extern Protegrin-1 hinzugefügt, jedoch nicht kontinuierlich, weshalb die Reduktion der Krankheitserreger als nur kurzfristig gesehen werden darf. Dennoch bietet dieser Effekt neben dem Vorteil der bisher nicht und als extrem unwahrscheinlich einzustufenden Resistenzentwicklung, noch den Zeitgewinn in der Kolonialisation und Infektion, sowie die breite Wirksamkeit gegen die eingangs erwähnten Gram-negativen und Gram-positiven Bakterien, spezielle Pilze und je nach Aufbau der externen Membranen, auch pathogenen Viren. Diese Pluripotenz stehen im Experiment die Produktionskosten gegenüber.

Schlussfolgerung:

Wir konnten in unseren Studien zeigen, dass die Applikation eines Gemisches aus Protegrin-1 (einem kommerziell erhältlichen antimikrobiellen Peptid) und einem kommerziell erhältlichen Gewebekleber die bakterielle Keimbesiedelung in Verbrennungswunden signifikant zu reduzieren vermochte. Beide Komponenten erwiesen sich in unseren Händen als stabil und deaktivierten sich nicht gegenseitig. Eine Behandlung im Rahmen der klinischen Umsetzung an Verbrennungspatienten könnte kombiniert mit Hauttransplantaten und künstlichem Hautersatz Verwendung finden, um in der im Outcome so wichtigen Frühphase nach Trauma eine topische Unterstützung im Kampf gegen Krankheitserreger bereitzustellen. Weitere Anwendungsbereiche außerhalb der Verbrennungsmedizin sind denkbar. Ergänzende, vor allem aber klinische Studien lassen weitergehende Kenntnisse über diese natürlichen Bausteine des angeborenen Immunsystems erwarten.

Literaturverzeichnis:

1 Zasloff M. Antimicrobial peptides of multicellular organisms. Nature 2002; 415: 389-95

2 Bals R. [Antimicrobial peptides and peptide antibiotics]. Med Klin 2000; 95: 496-502

3 Thompson DF, Davis TW. The addition of antibiotics to fibrin glue. South Med J 1997; 90: 681-4

4 Osada T, Yamamura K, Yano K, Fujimoto K, Mizuno K, Sakurai T, Nabeshima T. Distribution and serum concentration of sisomicin released from fibrin glue-sealed dacron graft in the rat and human. J Biomed Mater Res 2000; 52: 53-7

5 Patrlj L, Kocman B, Martinac M, Jadrijevic S, Sosa T, Sebecic B, Brkljacic B. Fibrin glue-antibiotic mixture in the treatment of anal fistulae: experience with 69 cases. Dig Surg 2000; 17: 77-80

6 Qu XD, Harwig SS, Shafer WM, Lehrer RI. Protegrin structure and activity against Neisseria gonorrhoeae. Infect Immun 1997; 65: 636-9

7 Steinberg DA, Lehrer RI. Designer assays for antimicrobial peptides. Disputing the „one-size-fits-all" theory. Methods Mol Biol 1997; 78: 169-86

8 Gilpin DA. Calculation of a new Meeh constant and experimental determination of burn size. Burns 1996; 22: 607-11

9 Ganz T. Antimicrobial proteins and peptides in host defense. Semin Respir Infect 2001; 16: 4-10

10 Nizet V, Ohtake T, Lauth X, Trowbridge J, Rudisill J, Dorschner RA, Pestonjamasp V, Piraino J, Huttner K, Gallo RL. Innate antimicrobial peptide protects the skin from invasive bacterial infection. Nature 2001; 414: 454-7

11 Lehrer RI, Ganz T. Defensins of vertebrate animals. Curr Opin Immunol 2002; 14: 96-102

12 Zanetti M, Gennaro R, Romeo D. Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain. FEBS Lett 1995; 374: 1-5

13 De Y, Chen Q, Schmidt AP, Anderson GM, Wang JM, Wooters J, Oppenheim JJ, Chertov O. LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. J Exp Med 2000; 192: 1069-74

14 Agerberth B, Charo J, Werr J, Olsson B, Idali F, Lindbom L, Kiessling R, Jornvall H, Wigzell H, Gudmundsson GH. The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations. Blood 2000; 96: 3086-93

15 Ganz T, Metcalf JA, Gallin JI, Boxer LA, Lehrer RI. Microbicidal/cytotoxic proteins of neutrophils are deficient in two disorders: Chediak-Higashi syndrome and „specific" granule deficiency. J Clin Invest 1988; 82: 552-6

16 Steinstraesser L, Klein RD, Aminlari A, Fan MH, Khilanani V, Remick DG, Su GL, Wang SC. Protegrin-1 enhances bacterial killing in thermally injured skin. Crit Care Med 2001; 29: 1431-7

17 Chalekson CP, Neumeister MW, Jaynes J. Treatment of infected wounds with the antimicrobial peptide D2A21. J Trauma 2003; 54: 770-4

18 Milner SM, Cole A, Ortega MR, Bakir MH, Gulati S, Bhat S, Ganz T. Inducibility of HBD-2 in acute burns and chronic conditions of the lung. Burns 2003; 29: 553-5

19 Milner SM, Bhat S, Buja M, Gulati S, Poindexter BJ, Bick RJ. Expression of human beta defensin 2 in thermal injury. Burns 2004; 30: 649-54

20 Chen J, Falla TJ, Liu H, Hurst MA, Fujii CA, Mosca DA, Embree JR, Loury DJ, Radel PA, Cheng Chang C, Gu L, Fiddes JC. Development of protegrins for the treatment and prevention of oral mucositis: structure-activity relationships of synthetic protegrin analogues. Biopolymers 2000; 55: 88-98

21 Nishimoto K, Yamamura K, Fukase F, Kobayashi M, Nishikimi N, Komori K. Subcutaneous tissue release of amikacin from a fibrin glue/polyurethane graft. J Infect Chemother 2004; 10: 101-4

22 Marone P, Monzillo V, Segu C, Antoniazzi E. Antibiotic-impregnated fibrin glue in ocular surgery: in vitro antibacterial activity. Ophthalmologica 1999; 213: 12-5

23 Fujimoto K, Yamamura K, Osada T, Hayashi T, Nabeshima T, Matsushita M, Nishikimi N, Sakurai T, Nimura Y. Subcutaneous tissue distribution of vancomycin from a fibrin glue/Dacron graft carrier. J Biomed Mater Res 1997; 36: 564-7

Chapter 6.1

English summary

6

Infected burn wounds pose a source of high morbidity and mortality, and within infections, Gram-negative ones cause more serious problems than Gram-positives. The way by which living bacteria in a *per se* problematic deep partial thickness burn wound have an impact on the innate immune system remains rather unclear. Therefore, the goal of the research described in this thesis, was to shed more light onto the interaction of parts of the innate immune system with invading Gram-negative bacteria in deep partial thickness burns *in vivo* and the role of LBP in this and to investigate potential new therapeutic approaches to counteract Gram negative infections of burn wounds.

Lipopolysaccharide binding protein (LBP) is believed to play a key role in repelling invading Gram-negative bacteria as described in bacterial peritonitis in the past, and in initiating the host's immune response. We were therefore interested in the topical and systemic innate immune response generated by invading Gram-negative bacteria represented by a multiresistant *Pseudomonas aeruginosa* that was the source of delivering lipopolysaccharide (LPS) to the wound. The resulting LBP-LPS binding, mediated by several proteins such as membrane-bound and soluble DC14 and the Toll-like receptors, is believed to initiate the production of pro- and anti-inflammatory cytokines moderating the host´s mechanisms of defense. By means of LBP-knockout mice the unique opportunity was given to gain more insight into the innate immune system lacking the capacity of producing LBP in response to burn wound infliction, even without introducing a standardized infection. We furthermore designed an adenoviral construct encoding for LBP that was used for gene transfer studies to analyze the effect of LBP rescue in LBP knockout (LBPko) mice, respectively overexpression in wild type (wt) mice. Therewith we could possibly identify its potential as gene therapy tool for future application in burn wound treatment.

Recently, antimicrobial peptides were reported to play a crucial, but poorly understood and sometimes contradictory role in innate immunity. These peptides are usually produced following a 'supply and demand' situation by the organism in a state of inflammation or infection. Since they represent a new class of antimicrobial agents targeting bacteria at their outer cell wall composition with broad Gram-positive, Gram-negative and fungal activity, we were interested in integrating these newly found and promising peptides into our studies on burn wound infection treatment.

With regard to the clinical problems associated with infected burns potentially leading to high morbidity and mortality, a set of experiments was designed to address the following research aims:

1) *To develop an in vivo, deep partial thickness (IIB) burn wound model in mice and apply this to both a new LBP-knockout mouse model and its corresponding wild type control;*

2) *To gain more insight into the ability of LBP to reduce the number of living, multiresistant, Gram-negative bacteria in deep partial thickness (IIB) burn wounds with special regards to innate immunity;*

3) *To apply an adenoviral construct encoding LBP as a tool to study whether this intradermal adenoviral gene delivery system represents a feasible therapeutic approach to bacterial infected burns;*

4) *To test the ability of a new class of antimicrobial agents (AMPs) capable of reducing the number of bacteria in our deep partial thickness burn wound model.*

First, an *in vivo* burn wound model had to be established, enabling us to design and execute experiments aimed at gaining more insight into the complex immunological mechanisms occurring in type IIB, infected partial thickness burns. Since our lab had an LBP-knockout mouse colony available, whose feature was the inability to produce LBP, the first aim was to compare the ability of this colony to defend the host against standardized Gram-negative infections and compare this to wild type controls. For this purpose, we inflicted a defined burn wound to both animal groups. The burn itself was designed to feature a living, thus regenerating wound bottom, clinically defined as class IIB that struggles with the restoration of missing dermal structures *per se*. As learned from devastating patient courses on intensive-care burn units, IIB-burn wounds become even more deleterious when infected. Based upon the fact that some bacteria such as *Pseudomonas aeruginosa* hide in biofilm formations, thus making wound clearance even more difficult, we deliberately chose to culture a live, and above all, *multiresistant Pseudomonas aeruginosa* strain to be used in all our experiments. Pseudomonas *aeruginosa* continues to be one of the most problematic bacterias in burn wound care.

In **chapter 2** we described the design of the experimental set-up, consisting of the mouse colonies and burn wound challenge with standardized Gram-negative infection. At first, we were interested in finding out whether there were differences in *Pseudomonas* growth on burn wounds in either of the two mouse colonies. As a result, we did not find differences in bacterial growth, neither in knockout nor in wild-type mice. In order to study whether overexpression of LBP, either in wt or in LBP k.o. mice plays a key role in innate immunity in infected partial thickness burns, we designed an adenoviral construct encoding LBP. We showed that the LBP encoding virus was capable of inducing LBP protein expression in *in vitro* cell systems. Immunohistochemically studying b-gal staining in the skin of mice locally treated with the adenovirus encoding βgal as control viral construct, next demonstrated feasibility of transgene expression *in vivo* using this gene transfer system. By restoring LBP intradermally in otherwise LBP-deficient and by over express-

ing in wild type animals, we could confirm our hypothesis that this protein is important in Gram-negative infection, demonstrated by the reduction of *Pseudomonas* bacterial numbers. Restoring LBP in LBP-deficient mice led to a reduction of bacterial numbers by 44-fold, and over-expressing this protein in wt animals led to a reduction of 4.9-fold. Furthermore, we showed that adenoviral LBP gene therapy in infected partial thickness burns in rodents was feasible.

In **chapter 3,** we focused in more detail on the intradermal alterations occurring in an organism lacking LBP protein. We were specifically interested in the transcriptional changes taking place at the site of injury, and therefore compared intradermal gene expression profiles following burns in either mouse strain. Commercially available microarray-chips were used for this purpose. Eighteen-hundred-and-two genes were at least two times up- or down-regulated in knockout animals compared to wild-types. The most extensive up-regulation occurred in growth-related oncogene-1 (GRO-1), a CXC-chemokine known for its capacity of neutrophil chemoattraction in rodents. Neutrophils are key cells in orchestrating the immune response. In our model, GRO-1 was up-regulated intradermally 53-fold in LBP-knockout mice at six hours post-burn. Along with this neutrophil chemoattractant GRO-1, changes in pro- and anti-inflammatory cytokine gene expression were of interest. Since the effector cells of GRO-1 protein are neutrophils, we conducted a quantitative analysis of peripheral leukocytes and their subsets in LBP-knockouts versus wild-types, first in uninjured animals. This analysis revealed no significant difference in the total number of peripheral white blood cells and their subsets between the two when unharmed. We repeated the blood analysis at 24 hours following the type IIB-burn in both animal groups. In LBP-knockouts, the total peripheral white blood cell counts, as well as the numbers of neutrophils and lymphocytes detected, increased significantly at 24 hrs, compared to the baseline reading at an uninjured state. In wild-types, in contrast, the total white blood cell counts, along with neutrophils and lymphocytes, were not significantly affected, as opposed to monocytes which showed an increase at 24 hours in type IIB burn compared to their levels at an uninjured state. We next aimed to determine the expression of GRO-1 along with those of other pro- and anti-inflammatory cytokines using quantitative reverse-transcription polymerase chain-reaction (qRT-PCR) at 24 hours. At this later time point, GRO-1 was less extensively expressed in knockout skin than in wt skin, while the other cytokines studied, i.c., IL-6, TNFa and IL-10, showed no significant difference between the two groups at 24 hours.

To gain further information about the role of the neutrophil in the processes under study and to follow up on the changes in expression profile in time described in chapter 3, we expanded the timeline of our study on cell and chemo-/cytokine behavior to 96 hours (**chapter 4**). Of the cytokines studied, GRO-1, TNFa and IL-10, showed a biphasic expres-

sion, meaning an increase at 24 hours after the injury compared to 6 hours, decreasing at 48 hours and increasing again at 96 hours in both animal groups. In contrast, IL-6 showed a monophasic profile, with almost 10-fold lower expression levels in knockout mice in the later time points, whereas TNFa and IL-10 did not show significance between the groups. With regard to deciphering a possible relation between GRO-1 regulation in time and neutrophil behavior in our experimental set up, we studied neutrophils intradermally and in the peripheral white blood cell pool. Peripheral neutrophil numbers over the time-course between 24 and 96 hours remained unchanged in LBP-knockout mice, in contrast to those in the wild types, which showed a peak at 48 hours. 96 hours post burn, LBPko mice had almost doubled their total WBC numbers in peripheral blood, and demonstrated lymphocytosis, whereas knockout mice had less monocytes at 48 hours after burn. In order to study neutrophil distribution in the skin, we applied a myeloperoxidase (MPO) assay at 24, 48 and 96 hours. Every time point showed higher MPO-levels in wild type skin, suggesting larger numbers and/or higher activation of intradermal neutrophils in wild type mice. The neutrophil numbers or their intradermal activation could not be associated with the intradermal GRO-1 expression kinetics found earlier.

Since LBP has proven importance in defined bacterial infections in our burn model, we were interested in the natural bacterial colonization of the healing skin following a type IIB burn in knockouts and wild types. This condition was created by leaving the burn wound uncovered, thus without any occlusive dressing, and without defined bacterial infection. At 6 days post trauma, there were more bacteria on wild-type skin than on knockout, and that the colonies were for the most part Gram-positive. Lack of LBP obviously changes the organisms' topical capacity of defending infections arising in a burn model leading us to conclude that there might be a compensatory immunological mechanisms, the nature of which is currently unknown, compensating for the absence of LBP.

Regarding increasing problems with multi-drug resistant bacteria, an ancient part of the innate immune system has drawn more and more attention in the last 10 to 15 years. Antimicrobial peptides (AMPs) are known in plants, cold-blooded animals and vertebrae as well as humans. Their potential use in a clinically applicable formulation was the subject of our research described in **chapter 5**. AMPs represent ancient weapons, triggered by infection and inflammation. These peptides target invading bacteria, viruses and fungi as long as they are coated by lipid layers as their outer cell walls, and they also seem to participate in orchestrating immune responses. AMPs therefore seem to play a key role in innate immunity. We hypothesized that such peptides may be advantageous in the treatment of burn wound infections. To test our hypothesis, an antimicrobial peptide of 18 amino-acids in length was produced, called Protegrin-1 (PG-1). It's *in vitro* efficacy was tested first by means of a radial diffusion assay against the *Pseudomonas* strain we

used for all our experiments. We showed that the bacteriotoxic effect was concentration-dependent, and that the effect did not fade when mixing the peptide with a commercially available fibrin sealant. The necessity of mixing these two components was born out of practical handling problems, since the dissolved bioactive peptide had a watery consistency and simply would drip from the burn wounds without staying long enough to deliver its antibacterial effect. We next applied this formulation in *in vivo* studies onto our standardized, infected burn wounds. Substantial decrease of multi-drug resistant *Pseudomonas* colonies in partial thickness burns was observed.

Concluding, we successfully developed a mouse burn-wound model grade IIB, and applied it to a mouse colony which lacks the ability of producing LBP, important in defending Gram-negative infections, and their wild type controls. With this model, we demonstrated that absence of LBP in knockout animals *per se* did not hamper a proper defense response against infection at the burn wound site. An adenoviral construct encoding LBP reduced the bacterial numbers in knockouts and wt mice significantly, by topically either restoring or overexpressing this protein. Intradermal gene-chip analysis and gene expression profiles demonstrated profoundly altered pro- and anti-inflammatory cytokine expression profiles at various time points following burn in the absence of LBP protein synthesis capacity. The chemokine GRO-1 was found to be intradermally up-regulated in knockout animals at an early time point. As GRO-1 is considered a major attractant for neutrophils, we next studied leukocyte and leukocyte subsets in peripheral blood, revealing different cellular immune reactions in mice lacking LBP and having received a 25% total body-surface partial thickness burn. However, no higher neutrophil numbers or neutrophil activation was found in knockout animals systemically or locally at the site of burn. Knockout animals had less topical bacteria when we observed natural dermal re-colonialisation showing Gram-positive bacteria 6 days after burn. This led us to the conclusion, that there potentially is a compensatory immunological mechanism in the absence of LBP. By the application of Protegrin-1 as a model antimicrobial peptide, mixed with fibrin glue, we were able to demonstrate its efficacy of reducing *Pseudomonas* bacteria in the type IIB burn model. This represents a potentially new treatment for infected burn wounds.

Chapter 6.2

Future perspectives

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Based on our findings during the course of these experiments, several conclusions suggest further research in the field of innate immunity and burn wound infections.
Intensive care problems such as ventilator-associated infections, nosocomial wound infections, the general topic of sterility, antibiotic-induced bacterial selection, questions of burn-induced hypermetabolism, compromised host defense as a consequence of severe burn injury, general immunodepletion, the correct timing of surgical interventions and the like, make intensive care in burn units highly dynamic, sometimes poorly predictable, but always challenging. In the struggle for the best possible treatment, intensivists and surgeons need to be closely collaborating. Critical decisions must be taken on a daily basis, and often therapeutic regimes need to be adopted to the patient´s needs and rapid changes. Currently, severe burn patients remain at high risk for local and systemic infections, responsible for the main cause of death among these patients (1, 2).

Future perspectives of burn wound infections:

Worrisome reports of multi-drug resistant pathogens, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter* spp., as well as various resistant fungal strains, raise concerns with regards to burn victims and are causes of high morbidity and mortality within this group (3). Over the last years, not only multi-resistance and *pre se* troublesome Gram-negative infections are feared for in serious burn wound infections, but also polymicrobial infections, such as combinations of Gram-positive, Gram-negative bacteria and fungi, pose increasing, serious threats (4-6). Next to bacteria, rising numbers of fungi found in burns contribute to the often deleterious consequences of these problematic wounds (7). A weakened, immune-compromised host following severe burn marks the predisposition, where opportunistic pathogens such as *Staphylococcus aureus*, *Enterococcus faecalis* and *Pseudomonas aeruginosa* transform into potentially deadly microorganisms (8).

Worldwide, a debate is ongoing about the fact, that there seem to be regional differences regarding definitions of 'THE major pathogen' responsible for burn wound infection, and ultimately morbidity and mortality. Multinational, comparative epidemiologic studies are still missing (9) to shed more light onto this problem. In a recent survey of 104 U.S. burn units with 44% of the centers responding (3), *P. aeruginosa* was still identified as the most prevalent Gram-negative pathogen, followed by *Acinetobacter baumannii* and *Enterococcus spp.* (10). In Asia the picture is slightly different, where *A. baumannii* and *Proteus mirabilis* are found to be the most common causes of burn infection, with *Pseudomonas aeruginosa* presenting itself in third place (3). In Europe, however, *P. aeruginosa* next to *Escherichia coli* remain the two most common pathogens found on burn wounds, each responsible for 13% of all Gram-negative infections (11). Given that these studies

were rather recently published, these data need to be accepted as currently valid, as opposed to data from 15 or 20 years ago, when discussing 'regional differences' of burn wound infections and possible therapeutic consequences. At first, in every burn unit, the most problematic and most prevalent pathogen needs to be identified and therewith its source. Having accomplished this step, eradication and prophylaxis protocols need to be set up. Since burn units are places where moist, warm climate conditions are required, *Pseudomonas aeruginosa* finds almost ideal growth conditions. We deliberately chose this bacterium in all our experiments for obvious reasons, as *Pseudomonas* was and still is a serious threat for wound infections on burn patients. There is a constant need for improvement in wound dressings, sterility, mandatory intravenous ports, ventilator equipment, and wound care products thriving to induce re-epithelialization, to keep the chance of bacterial infection and/or fungal super-infection as low as possible until wounds are closed and epithelialized. New antibiotic drugs, new treatment standards, new bioactive coatings of catheters, repellent surfaces of implants capable of stimulating ingrowth but repelling pathogens etc., remain challenges for researchers and industry. Better understanding of results derived from research projects such as ours, form the basis of interventional strategies to help conquer or even prevent burn wound infections. Antimicrobial peptides might help in these clinical problems, as discussed here.

Future perspectives of 'the neutrophil' in burns:

With the skin as the first line of defense gone, the organism has to rely on the innate immune system to face any invading pathogen. In conditions such as burns, macrophages and neutrophils typically orchestrate the immune response and mediate innate and adaptive immunity. Several studies have confirmed that as a consequence of thermal injury alone, neutrophil chemotaxis is compromised (12-18). In our research, the neutrophil was of high interest, since GRO-1, the murine neutrophil chemo-attractant and equivalent to human IL-8, was strikingly up-regulated in the skin of burned LBP-knockout mice.
There are differences in the generation of neutrophil data, since some publications use animal, others human studies, making comparability questionable especially when dealing with complex and highly dynamic immunological scenarios such as wound infections and host responses. One has to decipher clearly between murine- and human-derived findings and care must be taken when it comes to interpretation. Neutrophils and their subpopulations, as well as macrophages are no exception in this regard. Interestingly, two subtypes of murine neutrophils seem to play a role in the activation of two subtypes of macrophages. The activation is triggered by neutrophils releasing IL-10 and CCL2 (chemokine ligand-2, also known as monocyte chemotactic protein-1 (MCP-1))(19). In 2004, Tsuda and co-workers published that murine neutrophils (PMNs) differentiated into several subtypes (PMN I, PMN II and PMN-N), each responsible for different tasks (20). Neu-

trophils are known to orchestrate the immune response and are capable of further activating immunocompetent cells such as macrophages. In humans, according to Kobayashi et al (8), two different types of neutrophils are responsible for macrophage differentiation in an immunocompetent and active, or inactive form of macrophage. The activation of immunocompetent macrophages is accomplished by neutrophils expressing CCL2/MCP-1. This cytokine is not only present in mice, but also in humans (21). Glycyrrhizin, an active component of licorice roots, is a drug used in Japan for over 20 years with good clinical results, known to block CCL2 and therefore increase the host´s resistance against polymicrobial infections (19). Next to its effect on neutrophils and the cascade of activating a subtype of macrophages, interestingly, this drug also seems to play a role in restoring antimicrobial peptide production in burns (22).

Given the fact that neutrophils seem to be impaired in chemotaxis by burn injury alone (12), the neutrophil population in our experiments should be studied in more detail in future experiments. Since it is now known that there are subpopulations responsible for macrophage activation and recruitment, emphasis could be put on deciphering their role in our experimental setting. Possibly, answers could be generated with regard to the different distribution of neutrophils which we have found in LBP-knockout mice versus wild-types, as well as with regard to their distribution at different time points within the same groups. Therefore, a possible differentiation of a PMN-I and PMN-II neutrophil subtype as proposed by Tsuda and co-workers, possibly detectable in FACS analyses, should be evaluated in knockouts versus wild types of our model. This PMN-differentiation could potentially explain the neutrophil distribution in both our mouse populations. In addition, in future studies another focus should lie on CXCR2 neutrophil receptors in LBP-knockout mice as discussed in chapter 4, where we discussed receptor-ligand specificity and affinity. Results derived from these studies could potentially explain the findings of our MPO assay in combination with the GRO-1 expression and shed more light onto neutrophil chemoattraction and their spatiotemporal distribution. It is currently not known, which mechanism led to the reduction of Gram-positive bacteria found in our LBPko animals, when compared to wild types. Gram-positive bacterial clearance, even in a burn model, involves binding to a protein (LTA) to initiate a transmembrane signal transduction enabled by TLR2, which is, at least at the moment, not known to be associated with LBP and activation of the TLR4 receptor system. Potentially, LBP-deficiency triggers an immunologic mechanism, capable of compensation the absence of LBP.

On antimicrobial peptides:

As stated before, antimicrobial peptides (AMPs) are ancient weapons, part of the innate immune system, and possess broad antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi and viruses (23-26). According to the *Shai-Matsuzaki-Huang model* (25, 27-29), by lipid-protein interaction of the outer cell wall with the antimicrobial peptide, pores are formed within the cell wall allowing intra-extracellular ion-transit and thus cell death. Recent information points at the importance of physiologic salt concentration being a factor with direct impact on AMP activity (30).

Skin-derived AMPs are more than simple 'naturally occurring antibiotics', because they can also trigger chemotaxis, as well as angiogenesis, cell proliferation, and the production and release of cytokines, chemokines and inflammatory mediators, which are important features in the pathogenesis of skin diseases (30) and host defense. These microbicidal activities together with their immune modulating properties make AMPs interesting candidates for the development of novel therapeutic agents. Unfortunately, at the moment, there is not enough knowledge on how exactly they orchestrate the innate and adaptive immune system and how, for instance, the intracellular pathways of secondary messengers in immunocompetent cells are initiated. Therefore, stimuli and effectors are currently not fully understood.

If not replaced or produced, AMPs' biological effects last only briefly. In the clinic, there are situations where a short acting antibiotic effect is actually wanted, such as when using implants. Here, only short-term protection is needed, namely, when contamination is imminent or unavoidable, like for instance when skin inevitably touches an implant during placement. This is, for instance, regularly the case with silicone breast implants which are pushed through a small skin incision into the underlying tissue pocket, potentially leading to acute, sub-acute or slow implant-related infections. Over time, several vendors have consequently propagated coatings for silicone breast implants expressing advantages in ingrowth and antibacterial properties. At the moment intense research is conducted to improve antimicrobial properties of the implant-tissue layer, including in the field of plastic surgery, orthopedics, oro-maxillofacial surgery, ophthalmology and dermatology. One idea would be to enhance the bacterial clearance at the implant´s interface by use of AMPs, where currently a multi-disciplinary workgroup is set-up at UMCG in which the author of this thesis participates.

With regards to burn-wound infection as in our experimental setup, several researchers have reported beneficial effects brought about by these peptides (31-35). Earlier it was described that the concentration of antimicrobial peptides was reduced in tissues surrounding burn wounds, being in part responsible for the impaired host resistance against *Pseudomonas aeruginosa* (36). Interestingly, when the antimicrobial peptide-production in

a rodent infection model with *Pseudomonas aeruginosa* was pharmacologically enhanced, infection was less severe (22).
There are several scenarios imaginable to improve the effect of antimicrobial peptides in infected burns. Firstly, these peptides could be used topically and be mixed with a fibrin sealant (37), such as in our studies, avoiding repetitive use and thus potential side effects. Secondly, since sometimes vital intravenous lines need to be placed in or close to burn areas, it is of utmost importance to keep them as sterile and as resistant against biofilm formation and bacterial ingrowth along the catheter as possible. It would make sense to protect such a vital device that needs to penetrate the skin and tissues to be placed intravenously by a layer of an antimicrobial agent. Thirdly, to prevent fluid loss and loss of proteins together with mechanical stress shielding, dermal substitutes could be equipped with such AMPs, again acting topically and making replacement easy, in case they lose antibacterial potency or microbial adoption / resistance occurs. Given their advantage of broad antibacterial potency, these coatings do not need to be made according to the antibacterial spectrum targeted at, thus affecting Gram-positive and Gram-negative bacteria as well as fungi at the same time. Fourthly, the most sophisticated way of enhancing topical resistance would be to stimulate the local sources that produce these peptides 'on demand', as demonstrated by Yoshida et al who used Glycyrrhizin (22).
However, certain issues need to be addressed before these potentially new agents can be used in the clinic. For example, the signal transduction pathways by which these molecules contribute to both innate and adaptive immunity are not completely unraveled yet, therewith it is impossible to rule out any side-effects. The link between the innate and adaptive immune system in which AMPs play a role is currently unclear. Furthermore, in contrast to preliminary reports, it seems that there are resistances possible to topically administered AMPs, demonstrating that excessive use may lead to contrary effects (38). As AMPs also manifest diverse biological functions beyond microbial killing, their administration should be wisely regulated to avoid detrimental effects on host homeostasis (30). There are other potential issues to be thought of, including cytotoxicity (39, 40), skeletal muscle degeneration (41), possible tumor proliferation (42), possible rosacea (43), and chronic inflammation associated with their use. The antimicrobial peptide (ta) Lactoferrin is currently being investigated as an anticancer drug in clinical trials (44, 45), but seems to play a role in inducing anemia and is associated with renal cell carcinoma (46), as well as increasing bone formation systemically (47), hence limiting its use. Next to all these possible drawbacks, antimicrobial peptides are still quite expensive to make, and have a short life-span.

Regardless their potential disadvantages, AMPs present themselves with a lot of advantages. First and foremost, they are broadly acting against a wide variety of pathogens and they are only produced following a 'supply and demand' pattern, topically or sys-

temically. They seem to take part in orchestrating the immune response against invading pathogens. If their production can be stimulated or their down-regulation be blocked, the benefit seems obvious. Currently however, the system of AMPs interacting with the innate and adaptive immune system, of which they are an integral part, needs to be investigated further before they can be widely applicable as new antimicrobial drugs.

On LBP, shock and sepsis:

According to our experimental design, we focused on investigating more thoroughly the importance of LBP in Gram-negative infections in burns. Since then, the picture of LBP and the role of this protein in Gram-negative infection has evolved in more detail. Evaluating Gram-negative infections in LBP-knockout mice by means of an adenoviral construct was proven to be valid by Hemmila et al (48). Here, a systemic gene therapy approach is presented in LBP-knockout mice compared to wild-types, in a Gram-negative pneumonia model. It was found that the otherwise high lethality in knockouts could be reversed by systemic LBP-adenoviral gene therapy. In more detail, the authors showed that the cellular response of the host to LPS is mediated through stepwise interactions involving LBP, soluble and membrane-bound CD14, and MD-2, which facilitated the rearrangement of TLR4 transmitting the signal intracellularely (49).

A fact which is interesting in the detoxification process of LPS is it's link to lipoproteins, in other words, the fat-metabolism. LBP catalyzes the transfer of LPS into lipoproteins, thereby enhancing LPS detoxification (50, 51). During an acute-phase response to endotoxemia, a dramatic increase in circulating LBP is seen (52). At least in part, LBP is interacting with apolipoprotein-B and binding to LDL, and VLDL, enhancing the LPS-binding capacity of these lipoproteins, and serving as protection from the effects of LPS toxicity as such (50), integrating circulating LPS into the LDL/VLDL fraction (50, 53-55). LDL and VLDL form complexes with LPS and therefore imply an important role in the defense against bacteria and endotoxin (50), acting as buffers of LPS. Therefore, during infection, lipid metabolism is part of the defense mechanism as it neutralizes endotoxin (LPS) as well as viruses (56). Protection by lipoproteins from LPS toxicity was demonstrated *in vivo* and *in vitro* (55, 57-59) and binding of LPS to lipoproteins has been demonstrated before (60-62). Coupling LPS to lipoproteins reduces LPS toxicity (63), supported by the fact that hypolipidemia in animals result in higher LPS-induced lethality (55), and in humans low cholesterol levels predict an increased risk of death from infection (64). New data on plasma phospholipid-transfer protein (PLTP) suggests that increasing circulating levels decrease lethality in LPS-induced sepsis and shock in rodents (49). PLTP is shown to modulate the lipoprotein association and metabolism of LPS (65). Furthermore, it seems to play a crucial role in development of atherosclerosis by controlling plasma HDL levels (66) known to contribute to this common disease. HDL is shown to bind and neutralize LPS in the

course of inflammation and sepsis (67), making it a possible target of immunomodulation.

Less (or absent) circulating peripheral LBP, as in the knockout mouse-model, means that LPS needs to be 'detoxified' elsewhere, for instance in the lipoprotein pathway as described above. As a consequence higher rates of atherosclerosis should be seen in LBP-deficient mice potentially leading to atherosclerosis-related diseases. Further studies testing this hypothesis could be executed in LBPko mice, possibly with atherosclerosis inducing diets.

On the dual role of LBP, the concentration-dependent effects of LBP, CD14, and MD-2:

Current research puts LPS recognition and binding to LBP, as well as binding to its anchors such as membrane-bound or soluble receptors such as CD14 (68), in a different light. According to Gioannini and other researchers (69, 70), LBP acts in a dose dependent manner *in vitro* and in rodent studies (71). In high concentrations for instance, LBP is thought to be capable of binding LPS and triggering intracellular secondary messengers by bypassing the initiating complex (MD-2/TLR4) (72), which it does not do in low concentrations. This feature is called the 'dual role' of LBP and CD14. Activation of the organism by LPS therefore occurs at low concentrations as found in tissues, while inhibition of cell activation takes place at high concentrations, as present in plasma. Of note, the LPS receptor-activation sequence is enabled by LBP, CD14 and MD-2, but can also be inhibited exactly by those three proteins in a concentration dependent manner (71). At low levels, LBP can catalyze the transfer of LPS to CD14. Tobias and coworkers (73) reported that a selective blockade of CD14 is possible, thereby preventing activation. At higher concentrations, LBP aids in clearing LPS aggregates via a non-activating cellular pathway (72, 74, 75) and may interfere with LPS transfer from membrane-bound CD14 (mCD14) to MD-2 (76). Numerous plasma proteins are furthermore known to bind LPS and therefore prevent its coupling to MD-2/TLR4 (77). These include collectins (78), anti-endotoxin antibodies, and the neutrophil granule proteins bactericidal permeability increasing protein (BPI) (79), Lactoferrin (80), CAP18 (81) and lysozyme (82), as well as tissue factor pathway inhibitor (83).

Since there is no control over the actual LPS concentration that was administered through burn wound infection in the course of our mouse experiments, we have no way of judging whether the LPS-LBP interaction occurred at low or high concentrations, unfortunately. In other words, the animals may have been immunologically activated or not. Un-

fortunately, we have no information about the LBP concentration of our wt mice during the course of our experiments, which would help in better understanding the LBP effect.

Whenever we compare research data to clinical data, it is of the highest importance that the model used resembles the clinical scenarios as closely as possible. In research, reproducibility and comparability within the experimental setup are of importance as well. In all our experiments, we used age and weight-matched **female** mice. This made a comparison between the mouse populations possible, but it neglects the fact that there were no data generated in males. For adequate research data dealing with infection, inflammation, survival and morbidity (as discussed earlier), the model used should be matched in male **and** female, since there seems to be a gender difference as for instance in survival and sepsis (84-86), with better odds on the female side.

In summary, we have shown LBP's potential in reducing bacterial numbers in standardized, infected partial thickness burns. We furthermore showed that the absence of the potential to produce LBP has an effect on chemokine and cytokine production intradermally resulting from burns, and on cellular leukocytes and leukocyte subsets thereby altering topical immunity. Lastly, we demonstrated that the application of antimicrobial peptides in infected burns is possible. A number of important questions have been raised that remain to be answered with regard to the complexity of innate immunity and the role of LBP in these processes, the role of neutrophils in LBP-knockouts in response to burn, and of LBP on Gram-negative as well as Gram-positive infections in partial thickness burns.

References:

1. Muller MJ, Herndon DN. The challenge of burns. *Lancet* 1994;343:216-220.
2. D'Avignon LC, Hogan BK, Murray CK, et al. Contribution of bacterial and viral infections to attributable mortality in patients with severe burns: an autopsy series. *Burns* 2010;36:773-779.
3. Branski LK, Al-Mousawi A, Rivero H, et al. Emerging infections in burns. *Surg Infect (Larchmt)* 2009;10:389-397.
4. Greenhalgh DG, Saffle JR, Holmes JHt, et al. American Burn Association consensus conference to define sepsis and infection in burns. *J Burn Care Res* 2007;28:776-790.
5. D'Avignon LC, Saffle JR, Chung KK, et al. Prevention and management of infections associated with burns in the combat casualty. *J Trauma* 2008;64:S277-286.
6. Miranda BH, Ali SN, Jeffery SL, et al. Two stage study of wound microorganisms affecting burns and plastic surgery inpatients. *J Burn Care Res* 2008;29:927-932.
7. Sarabahi S, Tiwari VK, Arora S, et al. Changing pattern of fungal infection in burn patients. *Burns [Epub ahead of print]* 2011.
8. Kobayashi M, Tsuda Y, Yoshida T, et al. Bacterial sepsis and chemokines. *Curr Drug Targets* 2006;7:119-134.
9. Azzopardi EA, Azzopardi SM, Boyce DE, et al. Emerging gram-negative infections in burn wounds. *J Burn Care Res* 2011;32:570-576.
10. Hodle AE, Richter KP, Thompson RM. Infection control practices in U.S. burn units. *J Burn Care Res* 2006;27:142-151.
11. Guggenheim M, Zbinden R, Handschin AE, et al. Changes in bacterial isolates from burn wounds and their antibiograms: a 20-year study (1986-2005). *Burns* 2009;35:553-560.
12. Butler KL, Ambravaneswaran V, Agrawal N, et al. Burn injury reduces neutrophil directional migration speed in microfluidic devices. *PLoS One* 2010;5:e11921.
13. Warden GD, Mason AD, Jr., Pruitt BA, Jr. Evaluation of leukocyte chemotaxis in vitro in thermally injured patients. *J Clin Invest* 1974;54:1001-1004.
14. Kim Y, Goldstein E, Lippert W, et al. Polymorphonuclear leucocyte motility in patients with severe burns. *Burns* 1989;15:93-97.
15. Hasslen SR, Nelson RD, Ahrenholz DH, et al. Thermal injury, the inflammatory process, and wound dressing reduce human neutrophil chemotaxis to four attractants. *J Burn Care Rehabil* 1993;14:303-309.
16. Manktelow A, Meyer AA. Lack of correlation between decreased chemotaxis and susceptibility to infection in burned rats. *J Trauma* 1986;26:143-148.
17. Solomkin JS, Nelson RD, Chenoweth DE, et al. Regulation of neutrophil migratory function in burn injury by complement activation products. *Ann Surg* 1984;200:742-746.
18. Adams JM, Hauser CJ, Livingston DH, et al. Early trauma polymorphonuclear neutrophil responses to chemokines are associated with development of sepsis, pneumonia, and organ failure. *J Trauma* 2001;51:452-456; discussion 456-457.
19. Yoshida T, Tsuda Y, Takeuchi D, et al. Glycyrrhizin inhibits neutrophil-associated generation of alternatively activated macrophages. *Cytokine* 2006;33:317-322.

20. Tsuda Y, Takahashi H, Kobayashi M, et al. Three different neutrophil subsets exhibited in mice with different susceptibilities to infection by methicillin-resistant Staphylococcus aureus. *Immunity* 2004;21:215-226.
21. Sukhumavasi W, Egan CE, Denkers EY. Mouse neutrophils require JNK2 MAPK for Toxoplasma gondii-induced IL-12p40 and CCL2/MCP-1 release. *J Immunol* 2007;179:3570-3577.
22. Yoshida T, Yoshida S, Kobayashi M, et al. Pivotal advance: glycyrrhizin restores the impaired production of beta-defensins in tissues surrounding the burn area and improves the resistance of burn mice to Pseudomonas aeruginosa wound infection. *J Leukoc Biol* 2010;87:35-41.
23. Harder J, Bartels J, Christophers E, et al. A peptide antibiotic from human skin. *Nature* 1997;387:861.
24. Lehrer RI, Lichtenstein AK, Ganz T. Defensins: antimicrobial and cytotoxic peptides of mammalian cells. *Annu Rev Immunol* 1993;11:105-128.
25. Lehrer RI, Ganz T. Defensins of vertebrate animals. *Curr Opin Immunol* 2002;14:96-102.
26. Lehrer RI, Ganz T. Cathelicidins: a family of endogenous antimicrobial peptides. *Curr Opin Hematol* 2002;9:18-22.
27. Shai Y. Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by alpha-helical antimicrobial and cell non-selective membrane-lytic peptides. *Biochim Biophys Acta* 1999;1462:55-70.
28. Zasloff M. Antimicrobial peptides of multicellular organisms. *Nature* 2002;415:389-395.
29. Nizet V, Ohtake T, Lauth X, et al. Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature* 2001;414:454-457.
30. Niyonsaba F, Nagaoka I, Ogawa H, et al. Multifunctional antimicrobial proteins and peptides: natural activators of immune systems. *Curr Pharm Des* 2009;15:2393-2413.
31. Jacobsen F, Baraniskin A, Mertens J, et al. Activity of histone H1.2 in infected burn wounds. *J Antimicrob Chemother* 2005;55:735-741.
32. Milner SM, Bhat S, Buja M, et al. Expression of human beta defensin 2 in thermal injury. *Burns* 2004;30:649-654.
33. Chalekson CP, Neumeister MW, Jaynes J. Treatment of infected wounds with the antimicrobial peptide D2A21. *J Trauma* 2003;54:770-774.
34. Steinstraesser L, Tack BF, Waring AJ, et al. Activity of novispirin G10 against Pseudomonas aeruginosa in vitro and in infected burns. *Antimicrob Agents Chemother* 2002;46:1837-1844.
35. Schittek B, Paulmann M, Senyurek I, et al. The role of antimicrobial peptides in human skin and in skin infectious diseases. *Infect Disord Drug Targets* 2008;8:135-143.
36. Kobayashi M, Yoshida T, Takeuchi D, et al. Gr-1(+)CD11b(+) cells as an accelerator of sepsis stemming from Pseudomonas aeruginosa wound infection in thermally injured mice. *J Leukoc Biol* 2008;83:1354-1362.
37. Lahoda LU, Wang SC, Vogt PM. [A mixture of antimicrobial peptides and fibrin glue in treatment of partial-thickness burn wounds]. *Chirurg* 2006;77:251-256.
38. Perron GG, Zasloff M, Bell G. Experimental evolution of resistance to an antimicrobial peptide. *Proc Biol Sci* 2006;273:251-256.
39. Sawicki W, Mystkowska ET. Contraceptive potential of peptide antibiotics. *Lancet* 1999;353:464-465.
40. Quinn K, Henriques M, Parker T, et al. Human neutrophil peptides: a novel potential mediator of inflammatory cardiovascular diseases. *Am J Physiol Heart Circ Physiol* 2008;295:H1817-1824.

41. Yamaguchi Y, Nagase T, Tomita T, et al. Beta-defensin overexpression induces progressive muscle degeneration in mice. *Am J Physiol Cell Physiol* 2007;292:C2141-2149.
42. Haura EB, Zheng Z, Song L, et al. Activated epidermal growth factor receptor-Stat-3 signaling promotes tumor survival in vivo in non-small cell lung cancer. *Clin Cancer Res* 2005;11:8288-8294.
43. Yamasaki K, Di Nardo A, Bardan A, et al. Increased serine protease activity and cathelicidin promotes skin inflammation in rosacea. *Nat Med* 2007;13:975-980.
44. Parikh PM, Vaid A, Advani SH, et al. Randomized, double-blind, placebo-controlled phase II study of single-agent oral talactoferrin in patients with locally advanced or metastatic non-small-cell lung cancer that progressed after chemotherapy. *J Clin Oncol* 2011;29:4129-4136.
45. Kozu T, Iinuma G, Ohashi Y, et al. Effect of orally administered bovine lactoferrin on the growth of adenomatous colorectal polyps in a randomized, placebo-controlled clinical trial. *Cancer Prev Res (Phila)* 2009;2:975-983.
46. Loughlin KR, Gittes RF, Partridge D, et al. The relationship of lactoferrin to the anemia of renal cell carcinoma. *Cancer* 1987;59:566-571.
47. Cornish J, Callon KE, Naot D, et al. Lactoferrin is a potent regulator of bone cell activity and increases bone formation in vivo. *Endocrinology* 2004;145:4366-4374.
48. Hemmila MR, Kim J, Sun JM, et al. Gene therapy with lipopolysaccharide binding protein for gram-negative pneumonia: respiratory physiology. *J Trauma* 2006;61:598-605; discussion 605-596.
49. Gautier T, Klein A, Deckert V, et al. Effect of plasma phospholipid transfer protein deficiency on lethal endotoxemia in mice. *J Biol Chem* 2008;283:18702-18710.
50. Vreugdenhil AC, Snoek AM, van ,t Veer C, et al. LPS-binding protein circulates in association with apoB-containing lipoproteins and enhances endotoxin-LDL/VLDL interaction. *J Clin Invest* 2001;107:225-234.
51. Vreugdenhil AC, Snoek AM, van ,t Veer C, et al. LPS-binding protein circulates in association with apoB-containing lipoproteins and enhances endotoxin-LDL/VLDL interaction. *J Clin Invest* 2001;107:225-234.
52. Froon AH, Dentener MA, Greve JW, et al. Lipopolysaccharide toxicity-regulating proteins in bacteremia. *J Infect Dis* 1995;171:1250-1257.
53. Read TE, Harris HW, Grunfeld C, et al. The protective effect of serum lipoproteins against bacterial lipopolysaccharide. *Eur Heart J* 1993;14 Suppl K:125-129.
54. Auerbach BJ, Parks JS. Lipoprotein abnormalities associated with lipopolysaccharide-induced lecithin: cholesterol acyltransferase and lipase deficiency. *J Biol Chem* 1989;264:10264-10270.
55. Feingold KR, Funk JL, Moser AH, et al. Role for circulating lipoproteins in protection from endotoxin toxicity. *Infect Immun* 1995;63:2041-2046.
56. Grunfeld C, Marshall M, Shigenaga JK, et al. Lipoproteins inhibit macrophage activation by lipoteichoic acid. *J Lipid Res* 1999;40:245-252.
57. Flegel WA, Baumstark MW, Weinstock C, et al. Prevention of endotoxin-induced monokine release by human low- and high-density lipoproteins and by apolipoprotein A-I. *Infect Immun* 1993;61:5140-5146.
58. Harris HW, Grunfeld C, Feingold KR, et al. Chylomicrons alter the fate of endotoxin, decreasing tumor necrosis factor release and preventing death. *J Clin Invest* 1993;91:1028-1034.

59. Cue JI, DiPiro JT, Brunner LJ, et al. Reconstituted high density lipoprotein inhibits physiologic and tumor necrosis factor alpha responses to lipopolysaccharide in rabbits. *Arch Surg* 1994;129:193-197.
60. Ulevitch RJ, Johnston AR, Weinstein DB. New function for high density lipoproteins. Their participation in intravascular reactions of bacterial lipopolysaccharides. *J Clin Invest* 1979;64:1516-1524.
61. Ulevitch RJ, Johnston AR, Weinstein DB. New function for high density lipoproteins. Isolation and characterization of a bacterial lipopolysaccharide-high density lipoprotein complex formed in rabbit plasma. *J Clin Invest* 1981;67:827-837.
62. Van Lenten BJ, Fogelman AM, Haberland ME, et al. The role of lipoproteins and receptor-mediated endocytosis in the transport of bacterial lipopolysaccharide. *Proc Natl Acad Sci U S A* 1986;83:2704-2708.
63. Flegel WA, Wolpl A, Mannel DN, et al. Inhibition of endotoxin-induced activation of human monocytes by human lipoproteins. *Infect Immun* 1989;57:2237-2245.
64. Jacobs D, Blackburn H, Higgins M, et al. Report of the Conference on Low Blood Cholesterol: Mortality Associations. *Circulation* 1992;86:1046-1060.
65. Gautier T, Lagrost L. Plasma PLTP (phospholipid-transfer protein): an emerging role in 'reverse lipopolysaccharide transport' and innate immunity. *Biochem Soc Trans* 2011;39:984-988.
66. Kallio KA, Buhlin K, Jauhiainen M, et al. Lipopolysaccharide associates with pro-atherogenic lipoproteins in periodontitis patients. *Innate Immun* 2008;14:247-253.
67. Murch O, Collin M, Hinds CJ, et al. Lipoproteins in inflammation and sepsis. I. Basic science. *Intensive Care Med* 2007;33:13-24.
68. Gioannini TL, Teghanemt A, Zarember KA, et al. Regulation of interactions of endotoxin with host cells. *J Endotoxin Res* 2003;9:401-408.
69. Gioannini TL, Teghanemt A, Zhang D, et al. Isolation of an endotoxin-MD-2 complex that produces Toll-like receptor 4-dependent cell activation at picomolar concentrations. *Proc Natl Acad Sci U S A* 2004;101:4186-4191.
70. Thompson PA, Berbee JF, Rensen PC, et al. Apolipoprotein A-II augments monocyte responses to LPS by suppressing the inhibitory activity of LPS-binding protein. *Innate Immun* 2008;14:365-374.
71. Munford RS. Detoxifying endotoxin: time, place and person. *J Endotoxin Res* 2005;11:69-84.
72. Gegner JA, Ulevitch RJ, Tobias PS. Lipopolysaccharide (LPS) signal transduction and clearance. Dual roles for LPS binding protein and membrane CD14. *J Biol Chem* 1995;270:5320-5325.
73. Tobias PS, Tapping RI, Gegner JA. Endotoxin interactions with lipopolysaccharide-responsive cells. *Clin Infect Dis* 1999;28:476-481.
74. Weiss J. Bactericidal/permeability-increasing protein (BPI) and lipopolysaccharide-binding protein (LBP): structure, function and regulation in host defence against Gram-negative bacteria. *Biochem Soc Trans* 2003;31:785-790.
75. Gutsmann T, Muller M, Carroll SF, et al. Dual role of lipopolysaccharide (LPS)-binding protein in neutralization of LPS and enhancement of LPS-induced activation of mononuclear cells. *Infect Immun* 2001;69:6942-6950.
76. Thompson PA, Tobias PS, Viriyakosol S, et al. Lipopolysaccharide (LPS)-binding protein inhibits responses to cell-bound LPS. *J Biol Chem* 2003;278:28367-28371.
77. Chaby R. Lipopolysaccharide-binding molecules: transporters, blockers and sensors. *Cell Mol Life Sci* 2004;61:1697-1713.

78. van de Wetering JK, van Golde LM, Batenburg JJ. Collectins: players of the innate immune system. *Eur J Biochem* 2004;271:1229-1249.
79. Amura CR, Kamei T, Ito N, et al. Differential regulation of lipopolysaccharide (LPS) activation pathways in mouse macrophages by LPS-binding proteins. *J Immunol* 1998;161:2552-2560.
80. Wang D, Pabst KM, Aida Y, et al. Lipopolysaccharide-inactivating activity of neutrophils is due to lactoferrin. *J Leukoc Biol* 1995;57:865-874.
81. Larrick JW, Hirata M, Balint RF, et al. Human CAP18: a novel antimicrobial lipopolysaccharide-binding protein. *Infect Immun* 1995;63:1291-1297.
82. Ohno N, Morrison DC. Lipopolysaccharide interactions with lysozyme differentially affect lipopolysaccharide immunostimulatory activity. *Eur J Biochem* 1989;186:629-636.
83. Park CT, Creasey AA, Wright SD. Tissue factor pathway inhibitor blocks cellular effects of endotoxin by binding to endotoxin and interfering with transfer to CD14. *Blood* 1997;89:4268-4274.
84. Adrie C, Azoulay E, Francais A, et al. Influence of gender on the outcome of severe sepsis: a reappraisal. *Chest* 2007;132:1786-1793.
85. Schroder J, Kahlke V, Book M, et al. Gender differences in sepsis: genetically determined? *Shock* 2000;14:307-310; discussion 310-303.
86. Schroder J, Kahlke V, Staubach KH, et al. Gender differences in human sepsis. *Arch Surg* 1998;133:1200-1205.

Chapter 6.3

Nederlandse samenvatting

6

Geïnfecteerde brandwonden gaan gepaard met een hoge morbiditeit en mortaliteit. Wanneer er sprake is van een geïnfecteerde brandwond, veroorzaken Gramnegatieve bacteriën grotere problemen dan Grampositieve. Hoe levende bacteriën in een partiële dikte brandwond het aangeboren immuunsysteem beïnvloeden is niet duidelijk. Daarom is het doel van het onderzoek in dit proefschrift het bestuderen van de interactie tussen delen van het aangeboren immuunsysteem en gramnegatieve bacteriën in een muizenmodel van brandwonden van partiële dikte. Daarnaast werd de rol onderzocht die *Lipopolysaccharide binding protein* (LBP) hierbij speelt en een potentieel nieuwe methoden om infecties van brandwonden met Gramnegatieve bacteriën te beheersen.

LBP wordt gezien als een sleutelproteïne om invasieve Gramnegatieve bacteriën tegen te werken en een immuunreactie van de gastheer te initialiseren. Dit werd eerder ook bij bacteriële peritonitis beschreven. Wij waren geïnteresseerd in locale en systemische immuunreacties op Gramnegatieve bacteriën, en gebruikten hiervoor multiresistente *Pseudomonas aeruginosa* bacteriën die lipopolysaccharide (LPS) in de wond brengen. Men denkt dat de resulterende LPS-LPB verbinding, die gemedieerd wordt door meerdere eiwitten zoals membraangebonden en vrij voorkomende CD14 en Toll-like receptoren, de productie van pro- en anti-inflammatorische cytokines initieert en daarmee de afweerreactie van de gastheer verandert. Door de beschikking te hebben over LBP-knock-out muizen hadden wij een unieke mogelijkheid meer inzicht te krijgen in de reactie op LPS van het aangeboren immuunsysteem dat geen capaciteit tot LBP-productie heeft, met en zonder gestandaardiseerde infectie.

Daarnaast gebruikten we een adenovirale construct coderend voor LBP waarmee we genoverdrachtstudies uitvoerden om het effect van LBP-herstel in LBP-knockout (LBPko) muizen en over-expressie in normale, wild-type muizen (wt) te bestuderen. Hiermee konden wij het potentieel van LBP voor gentherapie en eventuele toekomstige applicaties voor brandwondbehandeling verhelderen.

Kort geleden werd beschreven dat antimicrobiële peptiden een cruciale, maar slecht begrepen en soms wat tegenstrijdige rol in het aangeboren immuunsysteem kunnen spelen. Deze peptiden worden gewoonlijk volgens een "*supply-and-demand*" situatie geproduceerd door een organisme dat lijdt aan een infectie of ontsteking. Aangezien deze peptiden een nieuwe klasse van antimicrobiële middelen vertegenwoordigen die bacteriën aan hun buitenste celwand aanvallen, en aangezien ze een brede werking tegen Grampositieve en Gramnegatieve bacteriën en schimmels hebben, waren wij geïnteresseerd om deze nieuwe en veelbelovende peptiden in ons infectiologische brandwondenonderzoek te integreren.

Vanwege de klinische problemen die met geïnfecteerde brandwonden geassocieerd zijn en vaak tot hoge morbiditeit en mortaliteit leiden, hebben wij een aantal experimenten ontwikkeld om daarmee de volgende studiedoelen te onderzoeken:

1) *Een in vivo muizenmodel ontwikkelen voor diepe, partiële dikte (IIB) brandwonden en dit toepassen op nieuwe LBP-knock-out muizen;*

2) *Meer inzicht verkrijgen in de mogelijkheid van LBP om het aantal levende, multiresistente, Gramnegatieve bacteriën in een diepe, partiële dikte (IIB) brandwond te reduceren, met speciale aandacht voor het aangeboren immuunsysteem;*

3) *Een adenoviraal construct implementeren welke codeert voor LBP, en onderzoeken of dit construct potentieel voor therapeutische doeleinden in bacterieel geïnfecteerde brandwonden toe te passen is;*

4) *De geschiktheid van een nieuwe klasse van antimicrobiële peptides (AMPs) testen en hun vermogen bepalen om het aantal aan bacteriën te reduceren binnen het model van de diepe, partiële dikte brandwond.*

Allereerst moest er een *in vivo* brandwondenmodel ontwikkeld worden, dat het ontwikkelen en uitvoeren van de vervolgexperimenten mogelijk maakte, met als doel meer inzicht in complexe, immunologische mechanismen van geïnfecteerde, type IIB brandwonden toe te laten.
Ons eerste doel was het vermogen te vergelijken van de LBP-knockoutmuizen ten opzichte van de normale muizen om zich te verdedigen tegen gestandaardiseerde Gramnegatieve infecties te bestuderen. Hiervoor creëerden wij in beide muizen groepen een gestandaardiseerde brandwond. De wond zelf was bedoeld om een levende, herstellende wondbodem te creëren, welke klinisch als een klasse IIB brandwond gedefinieerd wordt, die de verloren dermale structuren moet herstellen. Naast de bekende desastreuze gevolgen voor patiënten op brandwonden ICs, is bekend, dat IIB brandwonden tot nog slechter resultaten leiden, zodra ze geïnfecteerd raken. Omdat sommige bacteriën zoals *Pseudomonas aeruginosa* zich in biofilmformaties kunnen verbergen en daardoor moeilijk bestreden kunnen worden, hebben wij voor een cultuur van levende, en voor al multiresistente *Pseudomonas aeruginosa* bacteriën voor al onze experimenten gekozen. *Pseudomonas aeruginosa* blijft een van de meest problematische bacteriën in de behandeling van brandwonden.

In **hoofdstuk 2** hebben we het ontwerp van onze experimentele *set-up* beschreven, bestaand uit de muizenkoloniën en de gestandaardiseerde gramnegatieve brandwondeninfecties. Allereerst waren wij geïnteresseerd of er verschillen in groei van *Pseudomonas* bestonden tussen beide muizengroepen. Deze bleek er niet te zijn. Om te kunnen bestuderen of over-expressie van LBP in normale of LBPko muizen een rol speelt in het aangeboren immuunsysteem, hebben wij een adenovirus ontwikkeld dat co-

deert voor LBP. We konden aantonen dat het LBP-virus *in vitro* in staat was om LBP-proteïneproductie te induceren. Het immunohistochemische onderzoek van een viraal b-gal-kleurend controleconstruct toegediend in de huid van controle muizen liet vervolgens de uitvoerbaarheid van *in vivo* adenovirale transgen-expressie van ons systeem zien. Door LBP intradermaal te herstellen bij LBP-deficiënte muizen en door over-expressie bij, normale, of wild-type muizen, werd onze hypothese bevestigd dat dit proteïne belangrijk is bij Gramnegatieve infecties. Het herstel van LBP in LBP-deficiënte muizen leidde tot een 44-voudige reductie van het aantal *Pseudomonas* bacteriën, en door middel van over-expressie bij wild type muizen, tot een 4,9-voudige reductie. Bovendien konden wij aantonen dat adenovirale gentherapie in een model van een geïnfecteerde, partiële dikte brandwond bij kleine knaagdieren *in vivo* mogelijk is.

In **hoofdstuk 3**, focusten we in meer detail op de intradermale veranderingen van muizen die geen LBP-proteïne kunnen produceren. Wij waren vooral geïnteresseerd in de transcriptionele veranderingen die in de wond plaats vinden, en daarom vergeleken wij intradermale genexpressieprofielen middels micro-array technologie na het toebrengen van brandwonden in beide muizengroepen. Vergeleken met wild type muizen kwamen 1802 genen in de knockout muizen tenminste twee keer hoger dan wel lager tot expressie. Het gen dat het hoogst tot expressie kwam in knockout muizen was *growth-related-oncogene-1* (GRO-1), een CXC-chemokine dat bekend staat om het vermogen neutrofielen in knaagdieren aan te trekken. Neutrofielen zijn sleutelcellen in het reguleren van een immuunreactie. Binnen ons model kwam GRO-1 intradermaal 6 uur na het aanbrengen van de brandwond in LBP-knockout muizen 53-keer hoger tot expressie. Naast deze verandering in het neutrofielen chemoattractant gen GRO-1, waren veranderingen van pro- en anti-inflammatoire cytokines voor ons van belang. Tevens voerden we een kwantitatieve analyse uit van perifere leukocyten en hun subsets in niet verwonde LBP-knockout muizen versus de normale muizen. Deze analyse liet geen significante verschillen zien in beide groepen voor wat betreft het totaal aantal perifere witte bloedcellen. Vervolgens herhaalden we dit experiment 24 uur nadat in beide groepen een IIB brandwond aangebracht was. In LBP-knockouts was het totale aantal perifere leukocyten en het aantal neutrofielen en lymfocyten 24 uur na het trauma significant verhoogd, vergeleken met de niet verwonde LBPko muizen. Normale muizen lieten daarentegen geen verschil van beide tijdstippen in perifere leukocyten, neutrofielen en lymfocyten zien. Wel kon een verschil in monocyten aantallen gezien worden, die 24 uur na type IIB brandwond significant verhoogd waren vergeleken met de niet gewonde toestand. Daarnaast wilden wij weten wat de expressie was van GRO-1 tesamen met andere pro- en anti-inflammatoire cytokines 24 uur na het induceren van de brandwond. Dit hebben we geanalyseerd door middel van kwantitatieve *reverse-transcription polymerase chain-reaction* (qRT-PCR). Op dit latere moment kwam GRO-1 in de huid van knockout muizen minder sterk tot uitdruk-

king dan in de huid van wild type muizen. Daarentegen waren de bestudeerde cytokines IL-6, TNFa en IL-10 op 24 uur niet significant verschillend in beide groepen.

Om nog meer inzicht in de rol van neutrofielen in deze processen te krijgen, en de veranderingen in de gen-expressieprofielen beschreven in hoofdstuk 3 te kunnen vervolgen, verlengden we de tijd van onze studie naar cel- en cytokinegedrag naar 96 uur **(hoofdstuk 4)**. Van de bestudeerde cytokines lieten GRO-1, TNFa en IL-10 een bifasische expressie zien in beide diergroepen: een toename 24 uur na trauma vergeleken met 6 uur na trauma, een afname na 48 uur en een tweede toename na 96 uur. IL-6 daarentegen liet een monofasisch verloop zien, waarbij we bijna 10 keer minder expressie in knockout muizen vonden op de latere tijdstippen. TNFa en IL-10 lieten geen significante verschillen zien tussen de twee muizengroepen. Om een mogelijke relatie tussen GRO-1-regulatie en neutrofielengedrag in onze experimentele setting te vinden, bestudeerden we neutrofielen zowel intradermaal en in het perifere bloed. Perifere neutrofielenaantallen veranderden niet tussen 24 en 96 uur na het trauma in LBP-knockout muizen ten opzicht van de normale, wild-type muizen. De normale muizen lieten een verhoging zien na 48 uur. Zesennegentig uur na het optreden van de brandwond, hadden LBPko muizen hun totale aantal witte bloedcellen bijna verdubbeld, en was er sprake van een lymfocytose, terwijl knockout muizen een afname van monocyten toonden 48 uur na het letsel. Om de neutrofielenverdeling in de huid te kunnen bestuderen, voerden we een myeloperoxidase (MPO) assay uit 24, 48 en 96 uur na verwonding. Elk tijdstip liet hogere MPO spiegels zien in de huid van normale muizen, wat suggereert dat zich een hoger aantal, of sterker geactiveerde, neutrofielenpopulatie in deze groep bevindt. Het aantal neutrofielen of hun intradermale activatie kon niet met de eerder gevonden, intradermale GRO-1 expressie kinetiek gerelateerd worden.

Omdat LBP belangrijk bleek te zijn voor gecontroleerde bacteriële infecties binnen ons model, waren wij geïnteresseerd in de natuurlijke bacteriële kolonisatie van herstellende huid met een type IIB brandwond. Hiervoor hebben we in knockout- en normale muizen de brandwonden onbedekt gelaten, dus zonder occlusief verband, en er werd geen gecontroleerde bacteriële infectie geïnduceerd. Zes dagen na het trauma, vonden we meer bacteriën op de huid van normale muizen dan op die van knockout muizen. Deze kolonies waren voornamelijk Grampositief. Gebrek aan LBP verandert blijkbaar de lokale afweercapaciteit van een organisme tegen infecties die in een brandwond ontstaan. Daaruit kunnen we concluderen, dat er mogelijk een immunologisch, compenserend mechanisme bestaat, welke in staat is om de afwezigheid van LBP ten minste te compenseren.

Gezien de toenemende problematiek van *multidrug* resistente bacteriën, heeft een oud deel van het aangeboren immuunsysteem de laatste 10 tot 15 jaar meer en meer aandacht gekregen. Antimicrobiële peptiden (AMPs) komen bij planten, koudbloedige dieren

en vertebraten en ook bij mensen voor. Hun potentiële nut voor de kliniek was onderwerp van ons onderzoek beschreven in **hoofdstuk 5**. AMPs zijn oude verdedigingsmiddelen, die door infecties en ontstekingen *getriggerd* worden. Deze kleine peptiden vallen binnendringende bacteriën, virussen en schimmels aan, wanneer deze bekleed zijn met lipide-lagen aan hun buitenste celwand. AMPs spelen dus een cruciale rol in het aangeboren immuunsysteem. Onze hypothese was, dat dit soort peptiden van nut waren voor de behandeling van brandwondgerelateerde infecties. Om dit te testen, hebben we een antimicrobiëel peptide ter grootte van 18 aminozuren laten maken wat *Protegrin-1* (PG-1) wordt genoemd. De *in vitro* effectiviteit werd eerst door middel van een radiale diffusie analyse tegen *Pseudomonas aeruginosa* getest. We konden aantonen, dat het bacteriotoxisch effect concentratie-afhankelijk was en dat dit effect niet verloren ging, zodra we het peptide met een commercieel te verkrijgen fibrinelijm mengden. De noodzaak om de twee componenten te mengen kwam voort uit praktische problemen: het opgeloste bioactieve peptide had een waterachtige consistentie en druppelde simpelweg van de brandwond af, zonder lang genoeg *in situ* te blijven om het antibacteriële effect uit te oefenen. Vervolgens pasten wij dit peptide toe in *in vivo* studies, binnen het gestandaardiseerde, geïnfecteerde brandwondmodel. We konden hier een substantiële reductie van *Pseudomonas* bacteriën in de brandwonden mee bewerkstelligen.

Samenvattend hebben we een muizenbrandwondenmodel van een diepte IIB ontwikkeld, en dit vervolgens op een muizenkolonie toegepast, die geen mogelijkheid had tot productie van LBP, wat als belangrijk voor het afweren van Gramnegatieve infecties word gezien. Met dit model konden wij aantonen, dat afwezigheid van LBP in knockout muizen *per se* een adequate immuunreactie in een geïnfecteerde brandwond niet in de weg staat. Een adenovirale construct welke LBP codeert, was in staat brandwond gerelateerde *Pseudomonas aeruginosa* bacterie-groei in knockout- en normale muizen significant te reduceren. Intradermale genexpressie analyse liet substantieel verschillende pro- en anti-inflammatoire cytokine-expressieprofielen zien op verschillende tijdpunten na aanbrengen van de brandwond in aan- respectievelijk afwezigheid van LBP proteïne. Het intradermale chemokine GRO-1 werd op een vroeg tijdstip na trauma in LBP-knockout muizen tot overexpressie gebracht. Omdat GRO-1 een belangrijke factor voor het aantrekken van neutrofielen wordt gezien, bestudeerden we leukocyten en leukocyten-*subsets* in het perifere bloed, en vonden verschillende cellulaire immuunreacties van muizen die geen LBP konden maken in reactie op de gestandaardiseerde, partiële dikte brandwond. Er worden niet meer neutrofielen of verhoogde neutrofielen-activiteit - systemisch of lokaal - in dit brandwondmodel van *knockout* muizen gevonden. In *knockout* muizen werden lokaal minder bacteriën gezien als ze een natuurlijke dermale rekolonisatie van 6 dagen post-trauma ondergingen, deze waren voornamelijk Grampositief. Wij concludeerden, dat er in het geval van afwezigheid van LBP mogelijk een compensatoir immunologisch

mechanisme bestaat. Door het toepassen van Protegrine-1, een voorbeeld antimicrobiëel peptide, in een mengsel met fibrine-lijm, konden wij een reductie van *Pseudomonas aeruginosa* bacteriën binnen een type IIB brandwond aantonen. Dit is een potentieel nieuwe behandeling van geïnfecteerde brandwonden.

Addendum 1

Contributing authors

Research idea:
Stewart C Wang (SCW) and Lars-Uwe Lahoda, Grace L Su (GLS) (all University of Michigan (UofM), Ann Arbor)

Lab discussions:
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Grants, applications, certifications:
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Execution of experiments, data generation:
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Addendum 2

List of publications LU Lahoda, MD

Published peer reviewed articles:

1) Soft tissue expansion before total knee arthroplasty in arthrodesed joints; N.Mahomed, N.McKnee, P.Solomon, **L.U.Lahoda**, A.E.Gross; JBJS (B), 1994, 76-B, 88-90
2) The use of tissue expanders in the revision of a knee fusion to a total knee arthroplasty; Solomon P.R.,**Lahoda L.U.**,Mahomed N., Gross A.E.; University of Toronto, Medical Journal, Vol.70 No.1, 1992
4) Vergleich der parallel und divergent augmentierten Rekonstruktionen des vorderen Kreuzbandes; **Lahoda LU**, Wiener Klin. Wochenschrift 105(19):558-559, 1993
5) Scapulothorakale Dissoziation, Ein missed injury?; **Lahoda LU**, Kreklau B, Gekle C, Muhr G; Unfallchirurg 1998 Oct., 101(10):791-5
7) Ergebnisse posttraumatischer Ellbogengelenksarthrolysen, Eine prospektive Studie; **Lahoda LU**, Klapperich T, Hahn MP, Muhr G; Chirurg 1999 Nov., 70(11):1302-6
10) Die Differentialtherapie der Radiusköpfchenfraktur; MV.Meyer-Marcotty, **LU Lahoda**, MP Hahn, G Muhr, Unfallchirurg 2002, 105:532-539
15) Ambulante und kurzzeitstationäre Handchirurgie: Möglichkeiten und Grenzen; Das-Gupta K, **Lahoda LU**, Boorboor P, Vogt PM, Chirurg. 2004 Mar;75(3):257-64
18) The free „mutton chop" flap: a fascio-musculocutaneous flap for the reconstruction of the entire sacral and perineal area; Vogt PM, Kall S, **Lahoda LU**, Spies M, Muehlberger T; Plast Reconstr Surg. 2004 Oct;114(5):1220-4
19) Use of soleus muscle flaps for coverage of distal third tibial defects; Kauffman CA, **Lahoda LU**, Cederna PS, Kuyon WM; J Reconstr. Microsurg., 2004 Nov 20(8): 593-7
21) Plasma concentration of edothelin-1 after myocutaneous latissimus-dorsi-transplantation, role in reperfusion injury; Jokuszies A, Jansen V, **Lahoda LU**, Steinau HU, Vogt PM; Hand Mikro Plastische Chirurgie, 2005 (6), 37(3), 193-201
22) Gestielte Lappenplastiken für Schädelkalotte, Orbita, Sakrum und Kniebereich; PM Vogt, T Peters, P Brachvogel, E Rickels, **LU Lahoda**, A Jokuszies, M Spieß, Handchir Mikrochir Plast Chir 2005; 37
24) Breast cancer – plastic surgical strategies for the treatment of tumor infiltration of the thoracic wall and brachial plexus; Vogt PM, Busch K, Spies M, **Lahoda LU**, Kall S, Klima U, Jokuszies A, Zentralbl Gynakol 2005 (12) 127 (6): 407-11
25) Influence of transendothelial mechanisms on microcirculation: consequences for reperfusion injury after free flap transfer. Previous, current, and future aspects. Review.; Jokuszies A, Niederbichler A, Meyer-Marcotty M, **Lahoda LU**, Reimers K, Vogt PM. J Reconstr Microsurg. 2006 Oct;22(7):513-8.
26) Resection of infected achilles tendon : Results after soft tissue coverage without tendon reconstruction; Boorboor P, **Lahoda LU**, Spies M, Kuether G, Waehling K, Vogt PM. Chirurg. 2006 Dec;77(12):1144-1151.

27) A mixture of antimicrobial peptides and fibrin glue in treatment of partial-thickness burn wounds; **Lahoda LU**, Wang SC, Vogt PM. Chirurg. 2006 Mar;77(3):251-6
28) Letter to the editor. Scaphoidfrakturen beim Sportler; Knobloch K, **Lahoda LU,** Heckmann A, Vogt PM., Sportorthopädie Sporttraumatologie 2006;22,76.
29) The free latissimus dorsi flap as primary free flap and crane flap with secondary pedicled transfer; Peters T, Fischer P, **Lahoda LU**, Niederbichler AD, Alkandari Q, Vogt PM, J Reconstr. Microsurgery, 2008 Feb;24(2):73-7
30) Functional and oncological outcomes after limb-salvage surgery for primary sarcomas of the upper limb; Vogt PM, **Lahoda LU**, Spies M, J Plast Reconstr Aesthet Surg, 2009, 61, 382-387.
31) Fully 3-dimensional digitally planned reconstruction of a mandible with a free vascularized fibula and immediate placement of an implant-supported prosthetic construction; Schepers RH, Raghoebar GM, Vissink A, **Lahoda LU**, Van der Meer WJ, Roodenburg JL, Reintsema H, Witjes MJ. Head Neck. 2011 Oct 24. [Epub ahead of print]

Book chapters:

1) **Lahoda LU**: Verbrennungstherapie. Interdisziplinäres Handbuch (Bruck, Steen, Müller) Ecomed Vlg. Landsberg, erschienen 6/2002, Kapitel: Sepsis und Hämofiltration Brandverletzter (Vogt, Lahoda, Köller)
2) **Lahoda LU**: Jahrbuch der Chirurgie 2004 (Grundmann, Holzgreve) Biermann Verlag; Plastische Chirurgie, Kapitel 13 S.281 (Muehlberger, Fischer, Lahoda, Choi, Kall, Vogt)
3) **Lahoda LU**: Septische Knochen- und Gelenkschirurgie (Hendrich, Frommelt, Eulert) Springer 2004; Kapitel: Plastisch-chirurgische Behandlung von Weichteildefekten, S.76 (Vogt, Lahoda, Kall, DasGupta)
4) **Lahoda LU**, Bund T, Vogt PM, Buchkapitel in „Krupp/Rennekampff: Plastische Chirurgie: Nekrotisierende Fasziitis", 29.Ausgabe 2004, Kap. III-4
5) **Lahoda LU**, Bund T, Vogt PM, Buchkapitel„Nekrotisierende Fasziitis","Kursbuch ärztliche Begutachtung" Ludolph/ Lehmann /Schürmann, EcoMed, 8.Ausgabe 12/07, VI-1.3.11, 2007
6) **Lahoda LU**, in Vogt PM: „Praxis der Plastischen Chirurgie", Springer Verlag 2011 Kap. 24, S.183 „Lappenplastiken Leiste und Becken"
7) **Lahoda LU**, in Vogt PM: „Praxis der Plastischen Chirurgie", Springer Verlag 2011 Kap. 31, S.271 „Gutartige Tumoren der Haut, inklusive Nävi"
8) **Lahoda LU**, in Vogt PM: „Praxis der Plastischen Chirurgie", Springer Verlag 2011 Kap.32, S.279 „Maligne Tumoren"
9) **Lahoda LU**, in Vogt PM:„Praxis der Plastischen Chirurgie", Springer Verlag 2011 Kap.33, S.289 „Strahlenfolgen"
10) **Lahoda LU**, in Vogt PM:„Praxis der Plastischen Chirurgie", Springer Verlag 2011 Kap.84, S.811 „Fehlbildungen der Füsse"

Addendum 3

Professional CV Lars-Uwe Lahoda, MD

Dr.Lars-Uwe Lahoda was born on Feb 18th 1968 in Zell am See, Salzburg, Austria. He studied medicine in Vienna, where he submitted his doctoral thesis on anterior cruciate ligament reconstruction and the effect of 'through the condyle versus over-the top augmentation', with Prof.Dr.R.Schabus being his doctoral father. In 1993, he was a foreign medical student at Mount Sinai Hospitals (orthopedic surgery, Prof.Dr.Allan E Gross), University of Toronto, Canada. On June 3rd 1993 he graduated as a medical doctor at the University of Vienna, after having been awarded a performance scholarship by the Austrian Government during the last year of his studies.

A few weeks later, he began his residency at the general hospital of Zell am See and completed basic medical training (surgery, traumatology, internal medicine, ENT, pediatrics, neurology and gynecology), before he applied for a scholarship, granted by the Austrian Government enabling a 6 months stay in New York as a foreign medical resident at the orthopedic department of Columbia Presbyterian Medical Center, NYU (Chair Prof.Dr.Louis U. Bigliani). In Austria he completed his advanced trauma life support (ATLS) training in 1995 to become a certified emergency physician. Dr.Lahoda applied for a residency training program in surgical traumatology in Bochum, Germany after having returned to Europe in 1996 and received his specialty in surgery, emphasis on orthopedic traumatology with Prof.Dr.Gert Muhr in 2000. He spent several years training in intensive care as well as rotating half a year with Prof.Dr.Ulrich Steinau, head of the plastic surgical department and burn unit at the "Bergmannsheil", University of Bochum's trauma hospital. After having worked for one year as a board certified surgeon at "Bergmannsheil", he applied for an research fellowship (NIH grant) at the Trauma Burn Center of the University of Ann Arbor, Michigan, at Prof.Dr. Stewart Wang´s lab, conducting the research being the subject of this thesis. He also spent time with the plastic surgical department at the University of Michigan, (head: Prof.Dr.William Kuzon). During the time in Ann Arbor, Dr. Lars-Uwe Lahoda was participating in the National Car Crash Investigation Program (CIREN). In 2003 he returned to Germany and completed his training in plastic surgery at the University of Hannover (head: Prof.Dr.Peter Vogt), covering every aspect of plastic, hand and reconstructive surgery and burns. He became staff in 2004, passed the boards as a certified plastic, hand and reconstructive surgeon in 2006, had his German doctoral degree officially accredited by the University of Hannover in the same year, and passed the boards for hand surgery in 2008.

Since April 2008 Dr.Lahoda works as staff member and consultant at the University of Groningen, the Netherlands (Prof.Dr.Paul Werker) and acted as Chief of Clinic for two years. In 2009 he was invited as visiting professor at the Plastic Surgical Department in Ann Arbor, MI.

Dr.Lahoda´s professional interest lies in reconstructive surgery, traumatology, hand surgery, microsurgery, hand and neck reconstructions and intensive care medicine as well as breast reconstructions. His research interest lies in immunology, especially wound infections, furthermore in foreign body reactions associated with surface-modifications and alterations regarding ingrowth. He is a reviewer for 3 medical journals, has published journal articles and book-chapters, and has given more than 60 presentations worldwide. Dr.Lahoda has been and is an invited speaker at specialist´s meetings and holds currently full membership in 11 medical professional societies in Austria, Germany, the Netherlands and the USA. He is a board member of the International Confederation for Plastic, Reconstructive and Aesthetic Surgery (IPRAS) and is the European liaison of the Plastic Surgical Research Council (PSRC).

Addendum 4

Acknowledgments

As possibly true for everyone else who successfully completed his/her PhD-thesis, this work represents the result of 'blood, sweat'n tears'. All in all, more than 10 years of my life including the 2 research years in Michigan have been put together in this very booklet. When I left Germany in 2001 for Ann Arbor, next to quitting my job in Bochum, I also left behind 'my' world of trauma surgery and intensive care medicine which had determined me and had caught my focus for so many years.
I did not know what the future might bring at the time, nor where it might possibly be. My medical world had turned upside down. Shortly after starting my research, facing the challenge of a cutting edge lab, the Twin Towers in New York fell, a city I knew and lived in. This proud and strong country turned into chaos right before my eyes. I remember details of this day very clearly… June had called and made us aware of the fact that 'America was under attack' as she put it and we therefore had to 'postpone our dinner' that night. Sandra and I had no idea what had happened, since we neither had TV nor radio as we only were in the country for a short time. Even today, whenever there are television programs or documentaries reporting on '9-11', my throat tightens. Therefore, this period of my life stands for lots of changes and personal events, keeping my memory fresh. I am proud to have been (and still am) a member of the 'Michigan family' as Bill Kuzon once put it. In Ann Arbor I met outstanding people, saw researchers work and think in spheres 'beyond my comprehension', and even gotten in touch with colleagues who until today, admirably compete on the highest clinical and scientific levels, as represented for instance at PSRC or SIS-A meetings. Next to the job, there was little time for a private life. We have attended June and Matt's wedding, Geine and John's, have even been witnesses to Hanka and Richard's and made great friends with Sandy and Graham, as well as Anu and Rinki, and Lisa. Also, we organized a 'stretch-limo' tour for my dad who had turned 60, saw Pfizer move headquarters to Ann Arbor with great impact on the city, rode to Domino's Farm a few times, and finally… I decided to skip traumatology in favor of plastic surgery when the decision was made to return to Europe. This should only give a glimpse of what was going on at the time, but until today, mentioned yet again, it still has an impact on me. But, this is the place meant to thank people who have helped, accompanied and contributed.

First and foremost I want to thank my parents. Not only during my time in Michigan, but of course throughout my life, my parents have supported me in every thinkable way, positively criticized and stimulated me and my thoughts and served as 'good spirits' in so many ups and downs. They were and always are there when needed, they make me feel save and 'home'. They are the best people to share thoughts with and whose opinion I reflect and appreciate enormously. Without them I wouldn´t be who I am and where I am today. Vielen, vielen Dank!

Starting at the very beginning of my career, I would like to thank all of my 'mentors' even though some might not be aware to be serving as such. Rudi Schabus, for taking me as a 'doctorate candidate' at the end of my studies in Vienna, canalizing and stimulating my interest in sports injuries, especially knees. Hadn´t I had my first meeting with Rudi, the trauma surgeon at the University of Vienna 1 hour before meeting with Prof.Meissl, the plastic surgeon discussing the same subject, my career would surely have been totally different.

I´d like to thank the University of Vienna and Austrian Government for granting me a 'gifted scholarship' during my studies as well as 2 stipends enabling me to: 1) go to Toronto at the end of my studies (Allan E Gross, Orthopedics at Mount Sinai) and 2) to reside in New York for 6 months spending half a year on the very impressive shoulder surgery program led by Louis Bigliani at NYU, Columbia Presbyterian Hospital. Rudi, Herbert Resch (Salzburg, Austria), Allan Gross and Louis Bigliani need to be gratefully mentioned as they initiated my 'kicking off' and therefore left their footprints.

My first 'real' job in 1993 when I started as a resident has taught me invaluable treasures: thinking of WHAT to do WHEN and that medicine is no walk in the park. I started off in the hospital of Zell am See, my home. Being thrown into the water you sink or swim. In order to get accepted for training, I needed to start working without salary for an unknown period of time in order to proof my ambition to become a resident. Finally, I got accepted and joined their rotational program for almost 3 years as part of my basic medical training.

Gert Muhr in Bochum has surely made a thorough impression in my life. Both educationally and personally, I am amazed by how a single person managed to have such an impact. At the current stage of my career I understand his thoughts much better he used to share in short, unforgotten, sometimes 'snappy' statements and I am proud to have trained in his institution. He has left a 'mark on my forehead', even though I don´t do traumatology anymore and it would be a lie not to admit, that I every now and then miss it, since I learned so much. Lots of friendships date back to these 5 years, I recall numerous unforgettable situations, both personal and professional.

I am also very grateful to have worked with Peter Vogt who I met while in Bochum and who introduced me to plastic surgery. I was extremely impressed by Uli Steinau, Detlev Hebebrand and Frank Peter and of course Peter himself. Since then I regarded plastic, especially reconstructive surgery including hands, as a logic, advancing step forward, based on my previous training. Peter has influenced me personally as well as professionally a lot. He once put it that way: '…do you realize that you are standing on the shoulders of giants..?' meaning Uli Steinau and Gert Muhr. After having returned from the US, I gladly joined his service in Hannover, which he had just taken over and begun to build.

When thinking of the time 2001 to 2003 in the Trauma Burn lab of UofM Ann Arbor, Michigan, I would like to thank my PI Stewart Wang, Grace Su, Mark Hemmila, Sam Arbabi,

Mark Sochor (as old, new family), Mita Ghosh, Jianmin Sun, Nancy Gong, Aladdein Mattar, Ali Amenlari, Richard Klein, as well as Sandy Lemkin, Carla Kohoyda-Inglis and Sybil Boone, the Remick lab and all members of UMPIRE/CIREN. Also, the 'Michigan family of plastic surgeons', my friends Bill Kuzon (a real role model) and Paul Cederna (congrats!, my greatest respect!), who are absolute powerhouses both personally and professionally. It is now that I realize what 'Michigan family' means and how lucky I am to be affiliated, how honored and extremely privileged I am to know them and have seen them work, seen them conduct research and approach problems. Stewart as my PI enabled me to dive into basic science and research on a high level, representing the backbone of this booklet, for which I am grateful.

Groningen, the Netherlands, Paul Werker and his team have offered a new home. Thanks to them, I picked up a new language, made new friends, faced new challenges and learned many things, in short: I progressed, for which I am again very thankful! People in the North of the Netherlands are straightforward, a handshake means contract and the way they are reminds me of my Austrian home, mixed with international experience, not bad! I would like to thank Paul, who observes carefully, critically, balances distance and closeness and most of all, tries to positively stimulate and offers support. Paul agreed to let me write this booklet (saying for instance one word: …'focus'…), which means – in regards of what I wrote about my past– simply a lot to me, and offers closing a long, sometimes painful but also wonderful and somewhat turbulent book chapter. Thank you, Paul.

Ingrid Molema deserves my very special thanks, since without her and her constant 'quest for improvement' this would surely not have been possible at all. Ingrid has a gift: encouraging people to literally give their best, push to their boundaries and limits and therefore show the best of what one can do. This really means a great deal to me, since it has helped opening up my eyes, understanding and thinking like 'real' researchers and being very, very analytical. Every tiny little step along the way, I had to admit to myself that Ingrid´s points were very well made, generated by careful analysis, respectful and most of all: resulted in improvement in so many ways. Thank you so much, with my deepest respect and acknowledgment I have to say: it has been a privilege for me!

I would like to thank everyone at the University of Groningen, my fellow staff colleagues, all of our AIOSsen / residents (keep up the great work and most of all: stay curious! there is so much to learn, thank you for the privilege of working with you and sharing my knowledge with you), students who start their research work in our department, the supporting people of the department, everyone on the ward, the outpatient clinic, people I collaborate with as a plastic, reconstructive surgeon and everyone who has helped directly or indirectly in the completion of this booklet. I am also very grateful to the commission (Professors Loe de Leij, Henny van der Mei and Paul van Zuijlen) on positively judging this work.

I am very grateful to be able to learn continuously, a lot, from lots of people. Hardly ever, I have to admit, am I satisfied with second best in professional terms, which I expect from myself and I realize that this might sometimes be hard to understand. My ethical attitude comes from several different influences, too many and too personal to be mentioned here, they know who they are and they know I shall always be bound to them. Through my rather 'twisty' and somewhat unusual education, including jobs in 5 different countries, I have gotten insights into thought processes and strategies of dealing with each other which opened up my mind, ever since living in an international students hostel, the Albert-Schweitzer-Haus in Vienna. I consider myself as 'curious' and my task to help and serve those needing medical attention, with the little I know and the little I can do.

Along my way, there are numerous personal friends, family and relations who have supported, influenced and changed me to what I am today, all of whom deserve my deepest appreciation and thanks. My old friends Alex, Christian, Megan, Ruth, Karin, Yvi, Mirjam Neumann and Dietmar have supported me, shared time and joy and sometimes sad moments, thank you all for being there when needed! Also, my 'new friends' Harald, Sandy and Graham, Richard and Hanka, Susan and Erik, thanks for your support and wonderful friendship!
Marieke and Charmaine: both of you are much more than 'just my paranimfs' to me, as you know. It is great that within such a short time, we have become such great friends, are taking part in each other's lives and truly are some 'soul mates'. Thanks for your help, understanding and support, all the Port wine, the cooking, the 'gezelligheid', the hours at Minnar's, the great food, your Maltese and international spirits and 'het Nederlands meisje' in jou, Marieke. Great to dream together, build futures and rehearse, think, create and reflect. Love you all (including George of course!!!).
Last but not least!! To all my friends: thank you for being around when needed, thank you for being such great friends who I don´t see as much as I actually want to since you are spread all over the world, thank you for enabling me to continue chatting, contemplating and having fun right where we had left the last time, 'as if there was no time in-between' and thank you for being a part of me.